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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US92/09792 <b>(22) International Filing Date:</b> 13 November 1992 (13.11.92) <b>(30) Priority data:</b> 792,492 14 November 1991 (14.11.91) US <b>(71) Applicants:</b> REGENERON PHARMACEUTICALS, INC. [US/US]; 777 Old Saw Mill River Road, Tarrytown, NY 10591 (US). AMGEN, INC. [US/US]; 1840 Dehavilland Drive, Thousand Oaks, CA 91320 (US). <b>(72) Inventors:</b> SQUINTO, Stephen, P. ; 281 Birch Lane, Irvington, NY 10533 (US). IP, Nancy ; 23 Emery Drive, Stamford, CT 06902 (US). GIES, David ; 82 Ann Street, Fairfield, CT 06430 (US). YANCOPOULOS, George, D. ; 428 Sleepy Hollow Road, Briarcliff Manor, NY 10510 (US). HU, Shaw-Fen, Sylvia ; 3256 Peppermint Street, Newbury Park, CA 91320 (US).		<b>(74) Agents:</b> MISROCK, S., Leslie et al.; Pennie & Edmonds, 1155 Avenue of the Americas, New York, NY 10036 (US). <b>(81) Designated States:</b> AU, BB, BG, BR, CA, CS, FI, HU, JP, KR, LK, MG, MN, MW, NO, PL, RO, RU, SD, UA, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> EXPRESSION OF NEUROTROPHIC FACTORS WITH HETEROLOGOUS PREPRO REGIONS  <b>(57) Abstract</b>  The present invention relates to chimeric prepro proteins or prepro peptides comprising neurotrophic factors, and the use of such precursors and their nucleic acid sequences to produce proteins or peptides which have one or more biological activities of a neurotrophin. The chimeric prepro molecules provided by the present invention contain a heterologous prepro region fused to a mature neurotrophin sequence or biologically active portion or derivative thereof. The mature neurotrophin sequences that can be used according to the present invention are those of the NGF/BDNF family of homologous molecules including but not limited to NGF, BDNF, NT-3, and NT-4. Similarly, the prepro regions can be derived from those neurotrophin molecules of the NGF/BDNF family including but not limited to NGF, BDNF, NT-3, and NT-4.		

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EXPRESSION OF NEUROTROPHIC FACTORS WITH  
HETEROLOGOUS PREPRO REGIONS

1. INTRODUCTION

5           The present invention relates to the construction and expression in eukaryotic host cells of novel chimeric prepro proteins or prepro peptides expressing bioactive neurotrophic factors. The invention is based, in substantial part, on the  
10 discovery that chimeric prepro proteins or prepro peptides comprising the prepro region of a first neurotrophic factor fused to the mature protein, or portion thereof, of a second, different neurotrophic factor undergo efficient post-translational processing  
15 resulting in an increased level of expression of the bioactive second neurotrophic factor protein.

2. BACKGROUND OF THE INVENTION

2. 1. NEUROTROPHIC FACTORS

20           The development and maintenance of the nervous system depends on proteins known as neurotrophic factors. A neurotrophic factor is a cytokine, a protein which acts as a messenger and communicates with other cells in the ongoing  
25 coordination and regulation of biological functions. Neurotrophic factors promote the survival and/or differentiation of components of the nervous system. Widespread neuronal cell death accompanies normal development of the central and peripheral nervous  
30 systems, and apparently plays a crucial role in regulating the number of neurons which project to a given target field (Berg, D. K., 1982, Neuronal Development 297-331). Ablation and transplantation studies of peripheral target tissues during  
35 development have shown that neuronal cell death

results from the competition among neurons for limiting amounts of survival factors ("neurotrophic factors") produced in their projection fields. Important neurotrophic factors identified to date include nerve growth factor (NGF; Levi-Montalcini and Angeletti, 1968, Phys. Rev. 48:534); neurotrophin-3 (NT-3; Hohn et al., 1990, Nature 344:339; Maisonpierre et al., 1990, Science 247:1446), brain-derived neurotrophic factor (BDNF; Barde et al., 1982, EMBO J. 1:549), neurotrophin-4 (NT-4; Hallbook et al., 1991, Neuron 6:845-858), and ciliary neurotrophic factor (CNTF; Lin et al., 1979, Science 246:1023).

Neurotrophins are generally synthesized in vivo as "prepro" precursor proteins. The "prepro" region refers to the NH<sub>2</sub>-terminus of the precursor which is proteolytically removed during biosynthesis of the mature, biologically active form of the protein. The "pre" region refers to the signal sequence normally removed by proteolytic processing during translocation across the cell membrane to yield a "pro"-protein; the "pro" region is then removed by proteolytic processing to yield the mature form (see e.g., Darnell et al., 1990, Molecular Cell Biology 2d ed., Scientific American Books, pp. 650-657).

#### 2. 1. 1. NERVE GROWTH FACTOR

Nerve growth factor (NGF) is by far the most fully characterized of these neurotrophic molecules and has been shown, both in vitro and in vivo, to be essential for the survival of sympathetic and neural crest-derived sensory neurons during early development of both chick and rat (Levi-Montalcini and Angeletti, 1963, Develop. Biol. 7:653-659; Levi-Montalcini et al., 1968, Physiol. Rev. 48:524-569). Until recently, almost all studies of NGF had focused on its role in

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the peripheral nervous system, but it now appears that NGF also influences the development and maintenance of specific populations of neurons in the central nervous system (Thoenen et al., 1987, Rev. Physiol. Biochem. Pharmacol. 109:145-178; Whittemore and Seiger, 1987, Brain Res. Rev. 12:439-464).

The abundance of NGF protein in mouse submaxillary gland allowed the primary amino acid sequence to be determined by relatively conventional protein chemistry (Angeletti and Bradshaw, 1971, Proc. Natl. Acad. Sci. 68:2417-2420). The NGF gene has now been cloned from many species, including mouse (Scott et al., 1983, Nature 302:538-540, human (Uilrich et al., 1983, Nature 303: 821-825), cow and chick (Meier et al., 1986, EMBO J. 5:1489-1493), and rat (Whittemore et al., 1988, J. Neurosci. Res., 20:402-410) using conventional molecular biology techniques based on the availability of the protein sequence of mouse NGF to design suitable oligonucleotide probes.

The mouse NGF gene encompasses approximately 45 kb, containing several small 5' exons, with alternating splicing resulting in four distinct mRNA species (Serby, et al., 1987, Mol. Cell. Biol. 7:3057-3064). Two major transcripts result in a "long" and "short" NGF prepropeptide (Edwards, et al., 1986, Nature 319:784-787; Serby, et al., 1987, Mol. Cell. Biol. 7:3057-3064). The "short" precursor contains a conventional signal sequence (pre-region) at the NH<sub>2</sub>-terminus which flanks the pro-region. The "long" precursor contains an additional "pro-region" at its NH<sub>2</sub>-terminus (see e.g., Suter et al, 1991, EMBO J. 10:2395-2400, Figure 1). To date, no functional distinction between the "long" and "short" NGF prepro precursor has been elucidated. However, the shorter mRNA transcript is more abundant in most

tissue (Edwards et al., 1986, J. Biol. Chem. 263:6810-6815).

The biologically active form of mouse NGF is a 7S complex, comprising a dimer of a fully processed mature form of  $\beta$ -NGF along with two members of the kallikrein family of serine proteases, the  $\alpha$ -subunit and  $\gamma$ -subunit of NGF (Varon et al., Biochemistry 7:1296-1303; Mason et al., 1983, Nature 303:300-307). The translation, processing and secretion of the NGF precursor to form a biologically active form of NGF is well documented. Darling, et al. (1983, Cold Spring Harbor Symp. Quan. Biol. 48:427-433), on the strength of the reported cDNA sequence encoding mouse NGF (Scott, et al., 1983, Nature 302: 538-540), utilized an in vitro cell free translation system to identify key intermediates in the biosynthesis of the 7S complex of NGF. The signal sequence of the prepro NGF precursor is removed via proteolytic processing to yield a pro-NGF species of approximately 31 kD. The pro-region of the pro-NGF intermediate contains a pair of arginine residues known to be endoproteolytic processing sites. Proteolytic processing at either of these residues results in an additional major (21 kD) and minor (18.5 kD) intermediate species. The mature form of NGF can be proteolytically derived from either of the above-mentioned intermediate species. At some point in the biosynthesis of the mature form of NGF, a COOH-terminal dipeptide (arg-gly) is proteolytically released.

The  $\gamma$ -subunit has been shown in vivo to proteolytically cleave the pro-NGF precursor to the mature form of NGF (Edwards, et al., 1988, J. Biol. Chem. 263: 6810-6815). Attempts to mimic the process in vitro were unsuccessful, resulting in unfaithful processing of the pro-NGF precursor, presumably due to

aberrant folding of the in vitro translation product. Silen and Agard (1989, Nature 341:462-464) demonstrated that the pro region may facilitate proper folding of the  $\alpha$ -lytic protease precursor. Therefore, 5 the pro region of the NGF precursor may also be required for proper folding prior to endoproteolytic processing to the mature form and association into the biologically active 7S NGF complex. Support for this hypothesis is documented in Suter et al. (1991, EMBO 10 J. 10:2395-2400), who assigned functions for two partially conserved domains within the pro-region of NGF. Domain I was shown to be essential for NGF expression in COS cells. Additionally, Domain II, located in the NGF pro-region proximal to the mature 15 coding region, was found to be involved in proteolytic processing.

Endoproteolytic processing of pro-NGF in vivo has recently been shown to be controlled by the human fur gene product, a membrane associated 20 endoprotease sharing structural homology with the KEX2 gene, which encodes a yeast endoprotease (Bresnahan, et al., 1990, J. Cell Biol. 111:2851-2859).

Therefore, initiation of biosynthesis of the active form of mouse NGF involves the transcription of 25 the NGF gene and possible alternative splicing of the transcription product to generate mRNA's capable of translation of either a long or short NGF preproprecursor. The long or short prepro NGF precursor is subsequently subjected to a series of 30 endoproteolytic processing events, possibly induced by proper folding of the precursor via the structural characteristics of the pro-region, resulting in the mature form of NGF.



2. 1. 2. BRAIN-DERIVED NEUROTROPHIC FACTOR

Using pig brain as a starting material, Barde et al. (1982, EMBO J. 1:549-553) reported a factor, now termed brain-derived neurotrophic factor (BDNF), which appeared to promote the survival of dorsal root ganglion neurons from E10/E11 chick embryos. The neurotrophic activity was found to reside in a highly basic protein (isoelectric point, pI 10.1) which migrated during sodium dodecyl sulfate (SDS) gel electrophoresis as a single band of 12.3 kD. It was noted that the highly basic nature and molecular size of BDNF were very similar to the NGF monomer.

The cloning of the BDNF gene was first performed as described in copending U.S. Patent Application Serial Number 07/400,591, filed August 30, 1989, which is incorporated by reference in its entirety herein (see also PCT International Publication No. WO 91/03568, published March 21, 1991). Complete cDNA and/or genomic BDNF genes were cloned from a variety of species, including human, pig, rat, and mouse and the sequences of these genes were determined. Expression of recombinant BDNF was achieved in COS cells.

The first demonstration of neuronal specificity of BDNF distinct from that of NGF was the demonstration in vitro that purified BDNF supports the survival of 40-50% of sensory neurons dissociated from the neural placode-derived nodose ganglion of the chick embryo at E6, E9 or E12 (Lindsay et al., 1985, J. Cell. Sci. Supp. 3:115-129). NGF was without apparent effect on these neurons either by itself or in conjunction with BDNF. It was later shown in explant culture studies that BDNF appeared to support survival and neurite outgrowth from other neural

placode-derived sensory ganglia, including the petrosal, geniculate and ventrolateral trigeminal ganglia (Davies et al., 1986, J. Neurosci. 6:1897-1904), none of which have been found to be sensitive to NGF. In addition to its effects on cultured neurons from peripheral ganglia, BDNF was found to stimulate survival and neuronal differentiation of cells cultured from quail neural crest (Kalcheim and Gendreau, 1988, Develop. Brain Res. 41:79-86).

Two recent studies with BDNF (Kalcheim, et al., 1987, EMBO J. 6:2871-2873; Hofer and Barde, 1988, Nature 331:261-262) have, however, indicated a physiological role of BDNF in avian PNS development. In addition to its effect on peripheral sensory neurons of both neural crest and neural placode origin, BDNF has been found to support the survival of developing CNS neurons; Johnson et al. (1986, J. Neurosci. 6:3031-3938) presented data indicating that BDNF supports the survival of retinal ganglion cells cultured from E17 rat embryos.

In addition to its effects on the survival of developing neurons in culture, BDNF has been shown to have effects on cultured adult peripheral and central nervous system neurons.

Analysis of the predicted primary structure of mature BDNF has revealed a striking similarity to NGF; with only three gaps introduced into the NGF sequences to optimize matching, 51 identities are common to the various NGFs (from snake to man) and BDNF. Importantly, these identities include six cysteine residues.

### 2. 1. 3. NEUROTROPHIN-3

Another member of the neurotrophin family, termed neurotrophin-3, was discovered, and the NT-3

gene was cloned from mouse, rat, and human (see U.S. Patent Application Serial No. 07/490,004, filed March 7, 1990, incorporated by reference in its entirety herein; see also PCT International Publication No. WO 91/03569, published March 21, 1991). The overall structure of mature mouse NT-3 protein, consisting of 119 amino acids with a computed pI of about 9.5, was found to resemble that established for NGF and BDNF; a putative signal sequence of 18 amino acids (showing 5 and 9 amino acid identities with BDNF and NGF, respectively) appears to be followed by a prosequence of 121 amino acids (as compared with a prosequence of 103 amino acids in mouse NGF and a prosequence of 112 amino acids in mouse BDNF). A comparison between mature mouse NGF, BDNF, and NT-3 revealed 54 amino acid identities. All 6 cysteine residues, known in NGF and BDNF to be involved in the formation of disulfide bridges (Leibrock et al., 1989, Nature 341:149-152; Angeletti, 1973, Biochem. 12:100-115), are amongst the conserved residues. Similarly, mature rat NT-3 appears to share 57% amino acid homology with rat NGF, and 58% amino acid homology with rat BDNF; 57 of the 120 residues (48%) appear to be shared by all three proteins. Again, the six cysteine residues of rat NGF and BDNF were found to be absolutely conserved in rat NT-3, and regions of greatest homology between the three proteins appear to cluster around these cysteine residues.

In addition to the homology between NT-3, NGF, and BDNF within a species, a high degree of conservation in nucleic acid sequence was observed between rat and human NT-3 within the region encoding the mature polypeptide (119 amino acids). The deduced amino acid sequences of mature rat and human (as well

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as mouse NT-3) appear absolutely identical, reminiscent of the high degree of conservation of BDNF, which shows complete identity in the amino acid sequence of the mature polypeptide among rat, mouse, human, and pig. By contrast, the amino acid sequences of mature human NGF and rodent NGF (mouse or rat) differ by approximately 10 percent.

Studies of the neurotrophic activity of NT-3 have indicated that NT-3 is capable of promoting survival and neurite outgrowth of dissociated dorsal root ganglion neurons in culture. Furthermore, NT-3 was observed to promote neurite outgrowth from both nodose ganglion and sympathetic ganglion explants, whereas BDNF promoted outgrowth from nodose ganglion but not sympathetic ganglion, and NGF promoted outgrowth from sympathetic ganglion but not nodose ganglion explants. Therefore, NT-3 appears to have a broader specificity of action than either BDNF or NGF.

#### 2.1.4 NEUROTROPHIN-4

Neurotrophin-4 is a novel member of the NGF family that has recently been cloned and isolated (Hallbook et al., 1991, Neuron 6:845-858). PCR fragments corresponding to the NT-4 gene from Xenopus and viper were obtained, and a genomic Xenopus clone was subsequently isolated. Nucleotide sequence analysis of this clone revealed an open reading frame for a protein of 236 amino acids, with several structural features similar to those of NGF, BDNF and NT-3. These features include a putative amino-terminal signal sequence and a potential N-glycosylation site near a proteolytic cleavage site. As is true for NGF, BDNF, and NT-3, the entire Xenopus pre-pro-NT-4 protein is encoded in one single exon.

## 2.2 PRODUCTION OF NEUROTROPHINS

Various expression vectors and hosts have been utilized in attempts to produce recombinant neurotrophins.

5 All using animal cell expression systems (mammalian kidney cells), Liebrock et al. [Nature 341:149 (1989)] reported the expression of biologically active pig BDNF, and Rosenthal et al. [Neuron 4: 767 (1990)], Maisonnepierre et al. [Science 247:1446 (1990)] and Hohn et al. [Nature 344:339 (1990)] separately reported the expression of biologically active NT-3 of various species. In addition, Chan et al. [EP Publication No. 370171, published May 1990] reported the expression of  
10 biologically active mature human BDNF from insect cells by way of a baculovirus expression system.

Regarding microbial production of neurotrophins, Iwai et al. [Chem. Pharm. Bull. 34:4727 (1986)] reported the expression of synthetic "genes"  
20 for human NGF and a fusion thereof in E. coli. The product was only characterized by molecular weight, after treatment with a reducing agent, and there was no information regarding the presence of biological activity.

25 Dicou et al. [J. Neuroscience Res. 22:13 (1989)] reported the separate expression of mouse and hNGF fusions in E. coli. Dicou et al. (1989, J. Neurosci. Res. 22:13-19) fused the complete mouse prepro-nerve growth factor DNA sequence to the  
30 carboxyl terminus of the beta-galactosidase gene of Escherichia coli, and also fused a genomic DNA fragment corresponding to codons 11 to 106 of the human nerve growth factor gene to the fifth codon of the amino terminus of beta-galactosidase. Both  
35 bacterial vectors were associated with the expression

of large amounts of the chimeric proteins. Although after bacterial cell lysis most of the chimeric mouse prepro-nerve growth factor appeared to be insoluble, the majority of human chimeric beta-nerve growth factor seemed to exist in the supernatant. Neurotrophic activity was not reported.

Finally, Hu et al. [Gene 70:57 (1988); and Abstract 343.16 of the 20th Ann. Meeting of the Soc. for Neuroscience (1990)] reported expression of mouse NGF in E. coli.

### 3. SUMMARY OF THE INVENTION

The present invention relates to novel chimeric prepro proteins or prepro peptides comprising bioactive neurotrophic factors, and the use of such precursors and their nucleic acid sequences to produce proteins or peptides which have one or more biological activities of a neurotrophin. The chimeric prepro molecules provided by the present invention contain a heterologous prepro region fused to a mature neurotrophin sequence or biologically active portion or derivative thereof. The mature neurotrophin sequences that can be used according to the present invention are those of the NGF/BDNF family of homologous molecules including but not limited to NGF, BDNF, NT-3 and NT-4. Similarly, the prepro regions can be derived from those neurotrophin molecules of the NGF/BDNF family including but not limited to NGF, BDNF, NT-3 and NT-4. The invention is based, in substantial part, on the discovery that chimeric prepro proteins or prepro peptides comprising the prepro region of nerve growth factor fused to the mature portion of brain-derived neurotrophic factor (prepro NGF/BDNF) are more efficiently processed by a eukaryotic host cell than homologous prepro brain-

derived neurotrophic factor (prepro BDNF). It is further based on the discovery that stably transfected and amplified eukaryotic host cells expressing chimeric prepro NGF/BDNF secrete only the mature form of BDNF into the media. According to the present invention, the "long" or "short" prepro regions of NGF can be utilized in the construction of chimeric neurotrophic genes.

The invention is also based on the discovery that chimeric prepro proteins or prepro peptides comprising the prepro region of NT-3 fused to the mature coding region of brain-derived neurotrophic factor (prepro NT-3/BDNF) are more efficiently processed than homologous prepro brain-derived neurotrophic factor (prepro BDNF). It is further based on the discovery that stably transfected and amplified eukaryotic host cell expressing chimeric NT-3/BDNF secrete only the mature form of BDNF into the media.

The present invention provides for nucleic acids encoding chimeric neurotrophic prepro proteins or prepro peptides, and for methods of expressing these chimeric neurotrophic proteins and peptides by use of such nucleic acids.

#### 4. DESCRIPTION OF THE FIGURES

Figure 1. Polyacrylamide gel electrophoresis of recombinant BDNF, NGF, and chimeric precursor forms. Cell supernatants from metabolically labeled CHO-DG44 cells stably transfected with various constructs were resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. Lane 1: wild-type control CHO-DG44 cells. The following constructs were used: expression vector pCDM8 containing the human NGF gene (lane 2); short

prepro BDNF construct (lane 3); long prepro NGF/BDNF chimeric construct (lane 4). Lane 5: molecular weight markers.

Figure 2. Bioactivity of recombinant BDNF.

5 Crude supernatants from transfected CHO cell lines were assayed with embryonic (E8) chick dorsal root ganglia and neurite outgrowth was scored. Closed diamonds: cell line DGC-N/B-2.5-#23 (containing long prepro NGF/BDNF chimeric construct). Dotted squares:  
10 cell line DGZ1000-B-3-2.5 (containing short prepro BDNF construct).

Figure 3. Sequence of human BDNF cDNA (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2), and comparison of DNA sequences from pig (SEQ  
15 ID NO:3 and SEQ ID NO:4), rat (SEQ ID NO:5 AND SEQ ID NO:6), and chicken (SEQ ID NO:7 and SEQ ID NO:8). The figure shown is from PCT International Publication No. WO 91/03568, published March 21, 1991.

Figure 4. Nucleotide (SEQ ID NO:9) and  
20 deduced amino acid (SEQ ID NO:10) sequence of human NGF. -187 through -1 indicates the long prepro region. The sequence information is from EP Publication 121,338, published October 10, 1984, by Gray and Ullrich.

25 Figure 5. Aligned DNA sequences of the rat (SEQ ID NO:11) and human (SEQ ID NO:13) NT-3 genes. The predicted translation start site is indicated by "PREPRO--" and the predicted start of the mature NT-3 is indicated by "MATURE--". The mature rat (SEQ ID  
30 NO:12) and human (SEQ ID NO:14) NT-3 proteins have identical amino acid sequences whereas their prepro regions differ at 11 positions, which are underlined. The figure shown is from PCT International Publication No. WO 91/03569, published March 21, 1991.



Figure 6. DNA fragment 3 (SEQ ID NO:15 and 16), utilized in the NT-3/BDNF chimeric construction, corresponding to 35 amino acids of the NT-3 prepro region (SEQ ID NO:17).

5 Figure 7. Western blot analysis of  
conditioned media from CHO cell clones expressing  
either the original prepro BDNF (lane 2-11) or the  
chimeric prepro NT-3/BDNF (lane 12-19). Lane 1 was  
loaded with 450 ng of purified mature human BDNF.  
10 Lane 20 was loaded with prestained low molecular  
weight markers from BRL. Lane 8 and lane 16 represent  
non-producing clones from each transfection.

#### 5. DETAILED DESCRIPTION OF THE INVENTION

15 The present invention relates to novel  
chimeric prepro proteins or prepro peptides comprising  
bioactive neurotrophic factors, and the use of such  
precursors and their nucleic acid sequences to produce  
proteins or peptides which have one or more biological  
20 activities of a neurotrophin. The chimeric prepro  
molecules provided by the present invention contain a  
heterologous prepro region fused to a mature  
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or derivative thereof. The mature neurotrophin  
25 sequences which can be used according to the present  
invention are those of the NGF/BDNF family of  
homologous molecules including but not limited to NGF,  
BDNF, NT-3 and NT-4. Similarly, the prepro regions  
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30 the NGF/BDNF family including but not limited to NGF,  
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prepro proteins or prepro peptides comprising the  
prepro region of nerve growth factor and the mature  
35 portion of brain-derived neurotrophic factor (prepro

NGF/BDNF) or the prepro region of NT-3 and the mature portion of brain-derived neurotrophic factor (prepro NT-3/BDNF) are more efficiently processed by a eukaryotic host cell than homologous prepro brain-derived neurotrophic factor (prepro BDNF). It is further based on the discovery that stably transfected and amplified eukaryotic host cells expressing chimeric prepro NGF/BDNF or NT-3/BDNF secrete only the mature form of BDNF into the media. The post-translational processing of homologous prepro BDNF is highly inefficient. In contrast, a member of the same neurotrophin gene family, NGF, is efficiently processed. Only the mature bioactive form of NGF is secreted into the host cell media following transient transfection. The BDNF processing problem has carried through in the generation of stable host cell lines for the production of mature bioactive BDNF. The present invention provides a novel solution to this processing problem by expression of chimeric constructs, which in a specific embodiment contains the long prepro region of NGF fused in frame to mature BDNF and in another specific embodiment contains the prepro region of NT-3 fused in frame to mature BDNF.

The present invention provides for nucleic acids encoding chimeric neurotrophic prepro proteins or prepro peptides, and for methods of expressing these chimeric neurotrophic proteins and peptides by use of such nucleic acids.

Expression of nucleic acids encoding chimeric neurotrophic prepro proteins or prepro peptides according to the present invention provide significant advantages relative to the use of nucleic acids encoding homologous neurotrophic prepro proteins or prepro peptides. Production of chimeric neurotrophic prepro proteins or prepro peptides

provides for increased expression levels of the bioactive neurotrophic factor. This increased level of expression should additionally provide for better bioactive neurotrophic factor purification schemes in that contaminating unprocessed forms of the expressed neurotrophic factors are not apparent in the crude supernatants.

#### 5.1 THE EXPRESSION PRODUCTS OF THE PRESENT INVENTION

The bioactive proteins which can be obtained according to the present invention are the mature neurotrophic factors which are members of the neurotrophin gene family, or biologically active portions or derivatives thereof. The term "biologically active" as used herein refers to the ability to express one or more biological activities of the full-length mature neurotrophin. Such neurotrophins include but are not limited to mature BDNF, NT-3, NGF and NT-4 and such other members as are identified by those methods utilized to determine members of the neurotrophin gene family (e.g., using molecular probes, generated by PCR, corresponding to regions of homology within the family; see PCT Publication WO 91/03569).

The DNA coding sequences for various neurotrophin proteins, which can be expressed using the present invention, are available. See, Ullrich et al. (Nature 303:821 (1983); E.P. Publication 121,338, published October 10, 1984) regarding hNGF coding sequences and, e.g., Meier et al. (EMBO J. 5:1489 (1986)) and Schwarz et al. (J. Neurochem. 52:1203 (1989)) regarding NGF cDNAs from various other species; ATCC plasmid strain phBDNF-C-1 (Accession No. 4068) regarding a hBDNF cDNA clone and, e.g., Leibrock et al., infra, regarding a pig BDNF cDNA; and ATCC

plasmid strain pC8-hN3 (P1) (Accession No. 40765) regarding a human NT-3 cDNA clone and Maisonpierre et al. (Science 247:1446 (1990)) and Hohn et al. (Nature 344:339 (1990)) regarding NT-3 coding sequences from various other species. The cloning of the human (Rosenthal et al., Neuron 4:767 (1990)) as well as rat (Maisonpierre et al., infra) NT-3 genes has been reported. Furthermore, the nucleotide and amino acid sequences for BDNF are disclosed in PCT Publication WO 91/03568, published March 21, 1991 and copending U.S. application Serial No. 570,657 filed August 20, 1990; the nucleotide and amino acid sequences for NT-3 are disclosed in PCT Publication WO 91/03569 published March 21, 1991 and copending application Serial No. 570,189, filed August 20, 1990). In addition, nucleotide and amino acid sequences for BDNF (SEQ ID NO:1-8), NGF (SEQ ID NO:9-10), and NT-3 (SEQ ID NO:11-14) are presented in Figures 3, 4, and 5, respectively, herein.

In addition, a neurotrophin gene from any organism may be identified using the regions of homology shared by any two members of the BDNF/NGF/NT-3/NT-4 family of molecules using the methods set forth above. For example, and not by way of limitation, a novel neurotrophin may be identified and cloned by BDNF/NGF/NT-3/NT-4 synthesizing degenerate oligonucleotides corresponding to segments of protein sequences highly conserved between any two neurotrophins. These oligonucleotides can then be used as primers in polymerase chain reaction (PCR) with cDNA template prepared from cells suspecting of expressing the desired neurotrophin. The products of PCR can then be used as probes to permit cloning of complete cDNA and/or genomic genes, the sequences of which can be determined by standard methods. Novel

neurotrophins can be identified by selecting those containing, in addition to the sequences homologous to other known neurotrophins, sequences non-homologous to other known neurotrophins (e.g., at least six  
5 contiguous nucleotides in which at least two nucleotides differ). Similarly, oligonucleotides corresponding to sequences of a neurotrophin in one species can be used in PCR to generate probes to permit cloning of the neurotrophin gene from other  
10 species.

NGF and BDNF are basic proteins of approximately 120 amino acids that share about 50% amino acid sequence identity, including absolute conservation of six cysteine residues that, in active  
15 NGF, have been shown to form three disulfide bridges (Bradshaw, A., 1978, Ann. Rev. Biochem. 47:191-216; Leibrock et al., 1989, Nature 341:149-52). Comparison of the sequences of NGF from evolutionarily divergent species has revealed that the amino acids flanking  
20 these cysteine residues comprise the most highly conserved regions of the molecule (Meier et al., 1986, EMBO J. 5:1489-93; Selby et al., 1987, J. Neurosci. Res. 18:293-8). Strikingly, these are also the regions which are most similar between BDNF and NGF  
25 (Leibrock et al., 1989, Nature 341:149-52).

In a preferred aspect of the present invention, a mature human neurotrophin is produced by expression of a chimeric prepro molecule according to the present invention. In a specific embodiment, the  
30 chimeric prepro molecule is encoded by a nucleic acid containing the long prepro region of NGF fused in frame to the coding sequence for mature BDNF. In another embodiment, the chimeric prepro molecule is encoded by a nucleic acid containing the prepro region  
35 of NT-3 fused in frame to the coding region for mature

BDNF. In yet another embodiment, the long prepro region of NGF is fused in frame to the coding region for NT-3.

As discussed supra, no distinct biological significance between the "long" and "short" prepro region of the NGF precursor has been documented. In another specific aspect of the invention, either the "long" or "short" prepro region may be utilized in the construction of chimeric neurotrophic genes. One of ordinary skill in the art can utilize either a "short" NGF prepro region or a "long" NGF prepro region when constructing chimeric fusions of the present invention comprising an NGF prepro region.

The mature neurotrophin molecules which can be expressed as chimeric prepro precursors according to the present invention also include substantially equivalent sequences, and fragments or derivatives which are biologically active.

For example, the neurotrophin nucleic acid sequences can be altered by substitutions, additions or deletions that provide for functional molecules. Due to the degeneracy of nucleotide coding sequences, other DNA sequences that encode substantially the same neurotrophin amino acid sequence may be used in the practice of the present invention. These include but are not limited to nucleotide sequences comprising all or portions of the neurotrophin genes that are altered by the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change. Likewise, the neurotrophin proteins, or fragments or derivatives thereof, of the invention include, but are not limited to, those containing, as part of their primary amino acid sequence, altered sequences in which functionally equivalent amino acid residues are substituted for

residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Also included within the scope of the invention are neurotrophin proteins or fragments or derivatives thereof which are obtained through modification during or after translation, e.g., by glycosylation, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, acetylation, phosphorylation, reduction, cleavage, etc.

Additionally, a given neurotrophin sequence can be mutated in vitro or in vivo, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further in vitro modification. Any technique for mutagenesis known in the art can be used, including but not limited to, in vitro site-directed mutagenesis (Hutchinson, et al., 1978, J. Biol. Chem. 253:6551), use of TAB® linkers (Pharmacia), etc.

The present invention also relates to expression of the nucleic acids encoding chimeric prepro neurotrophin molecules, and recovery of the mature neurotrophin product.

5

#### 5.2. THE CONSTRUCTION OF CHIMERIC NEUROTROPHIC PREPRO PROTEINS OR PREPRO PEPTIDES

Nucleic acids encoding chimeric neurotrophic prepro proteins or prepro peptides may be constructed using standard recombinant DNA technology, for  
10 example, by restriction enzyme digestion and ligation of nucleic acid sequences which encode the desired prepro and mature regions. Alternatively, nucleic acid sequences may be constructed using chemical  
15 synthesis, such as solid-phase phosphoramidate technology. In preferred embodiments of the invention, polymerase chain reaction (PCR; Saiki et al., 1985, Science 230:1350-1354) may be used to accomplish splicing of nucleic acid sequences by  
20 overlap extension (Horton et al., 1989, Gene 77:61-68) and thereby produce nucleic acids encoding the chimeric neurotrophic prepro proteins or prepro peptides of the invention (see e.g., Section 6, infra).

25 In a preferred aspect, the nucleic acids of the invention are produced by use of two separate PCR reactions, each with a different template. By way of illustration, if an X-Y chimera is desired, PCR is first carried out with one template, for example, X,  
30 using a probe completely homologous to X, and a probe with a region homologous to X and a region homologous to Y. The PCR reaction product is then isolated and used as probe in a second PCR reaction, with Y as a template, and a second probe completely homologous to  
35 Y.



It may further be desirable to incorporate useful restriction endonuclease cleavage sites in the primers.

In addition, chimeric neurotrophic factors may be produced by one-step PCR utilizing three oligonucleotide primers. For example, a nucleic acid encoding at least a portion of a desired prepro region (X) may be ligated to a nucleic acid sequence encoding a mature neurotrophic protein or peptide (Y) by creating three oligonucleotide primers, one of which corresponds to a portion of the X sequence (the "X primer"), another which corresponds to a portion of the Y sequence (the "Y primer"), and a third which contains a portion of both X and Y sequences ("the XY primer"). These three oligonucleotides may be combined in a one-step PCR, it being desirable that the X and Y primers are present in greater amounts than the XY primer, for example, at a ratio of X:XY:Y of about 100:1:100. [The template utilized in the PCR may be a mixture of nucleic acids encoding the desired prepro region and the mature neurotrophic protein or peptide.] The position of the splice site is determined by the bridging nucleotide (e.g. the XY primer).

Amplification conditions routinely used in the art may be used, for example, 1 minute at about 94°C, 2 minutes at about 43°C and 3 minutes at about 72°C for 35 cycles, using standard PCR reaction solutions and methods. The resulting PCR fragment may then be gel purified using gel electrophoresis, digested with the appropriate restriction endonuclease and ligated into a suitable cloning vector.

Additional methods of constructing the chimeras of the present invention will be readily apparent to those skilled in the art.

DNA reaction products may be cloned using any method known in the art. Any number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, cosmids, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, or plasmids such as pBR322, pUC, or Bluescript® (Stratagene) plasmid derivatives. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc.

5.3. EXPRESSION OF NUCLEIC ACIDS ENCODING  
CHIMERIC NEUROTROPHIC PREPRO PROTEINS  
OR PREPRO PEPTIDES

The nucleotide sequence coding for a chimeric neurotrophic prepro protein or prepro peptide, can be ligated into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription of the cloned chimeric DNA sequence. The necessary transcriptional and translation signals can also be supplied by one of the neurotrophin genes and/or its flanking regions corresponding to the chimeric neurotrophic prepro protein or prepro peptide. A variety of eukaryotic host-vector systems may be utilized to express the cloned chimeric DNA sequence and resulting mRNA transcript. These include but are not limited to mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.), transfected with other vectors, containing chromosomally integrated nucleic acids of the invention, etc., but the host system used must have the appropriate cell machinery to process the prepro chimera to the mature neurotrophin. The expression elements of vectors vary

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in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.

5 Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a sequence encoding a chimeric neurotrophic prepro protein or prepro peptide, consisting of appropriate  
10 transcriptional/translational control signals upstream of the chimeric DNA sequences. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (genetic recombination). Expression of nucleic acid sequences  
15 encoding chimeric neurotrophic prepro protein or prepro peptide may be regulated by a second nucleic acid sequence so that chimeric neurotrophic prepro protein or prepro peptide is expressed in a host transformed with the recombinant DNA molecule. For  
20 example, expression may be controlled by any promoter/enhancer element known in the art to be active in mammalian cells. Promoters which may be used to control chimeric neurotrophic factor expression include, but are not limited to, the  
25 cytomegalovirus (CMV) promoter, the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine  
30 kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:144-1445), the regulatory sequences of the metallothioneine gene (Brinster et al., 1982, Nature 296:39-42); and the following animal transcriptional control regions, which exhibit tissue  
35 specificity and have been utilized in transgenic

animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58); alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

A specific example of an expression vector which can be used is CDM8 (Seed, 1987, Nature 329:840-842; Seed and Aruffo, 1987, Proc. Natl. Acad. Sci. USA 84:3365-3369; Aruffo & Seed, Proc. Natl. Acad. Sci.

USA 84: 8573-8577); another example being pCMX (see copending application Serial No. 678,408, filed March 28, 1991).

Expression vectors containing chimeric neurotrophic prepro protein or prepro peptide gene inserts can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to at least a portion of an inserted chimeric neurotrophic prepro protein or prepro peptide gene. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, transformation phenotype, etc.) caused by the insertion of foreign genes in the vector. For example, if the chimeric neurotrophic prepro protein or prepro peptide coding sequence is inserted within the marker gene sequence of the vector, recombinants containing the chimeric insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the neurotrophic factor gene product in bioassay systems as described infra, in Section 5.4.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established,

recombinant expression vectors can be propagated and prepared in quantity.

Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered chimeric neurotrophic prepro protein or prepro peptide may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g., glycosylation, cleavage) of proteins. Appropriate cell lines or host systems should be chosen to ensure the necessary processing (e.g., removal by cleavage of the prepro region) and any desired modification. Mammalian host cells, such as monkey, human, or bovine, are thus preferred.

In specific embodiments of the invention, DNA encoding chimeric neurotrophins may be expressed in a CHO cell system according to methods set forth infra. Once a recombinant which expresses the chimeric neurotrophin is identified, the mature gene product should be analyzed. This can be achieved by assays based on the physical or functional properties of the product. See infra Section 5.4.

Once the mature neurotrophic factor protein or peptide is identified, it may be isolated and purified by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

#### 5.4. NEUROTROPHIC FACTOR ASSAYS

The neurotrophin proteins and peptides produced according to the invention are able to exhibit one or more biological activities including

but not limited to neurotrophic activity, binding by antibodies to neurotrophins, binding to cognate receptors, etc. The term "neurotrophic activity", as used herein, should be construed to refer to a biological effect on nervous system cells, including, but not limited to, neurons, astrocytes, glial cells, oligodendrocytes, microglia and Schwann cells. The biological effect is an alteration in the structure and/or physiology of a nervous system cell which does not occur absent direct or indirect exposure to the chimeric neurotrophic factor. Examples of a biological effects are the prolongation of survival, neurite sprouting, the maintenance or development of differentiated functions (such as expression of an enzyme e.g. choline acetyltransferase or tyrosine hydroxylase) or, conversely, cell death or senescence, or dedifferentiation.

The presence of neurotrophic activity may be determined using any known assay for such activity as well as systems which may be developed in the future. Assay systems may include in vitro testing systems, such as tissue culture bioassay systems using tissue explants, cells prepared from tissue, or immortalized cell lines, for example, derived from the brain, spinal cord, or peripheral nervous system, as well as in vivo testing systems in which neurotrophic factor may be administered to an animal; neurotrophic effects may be detected in such an animal by performing, chemical, histologic, or behavioral tests using said animal. Additionally, a neurotrophic factor may be incorporated as a transgene in a non-human transgenic animal, and its biological effects may be measured in said animal.

For example, but not by way of limitation, neurotrophic activity may be measured using any of the following well known bioassay systems:

5 (i) dorsal root ganglia assay system, as described in Barde et al., 1980, Proc. Natl. Acad. Sci. USA. 77:1199-1203, which is incorporated by reference in its entirety herein;

10 (ii) nodose ganglia assay system as described by Lindsay et al., 1985, Dev. Biol. 112:319-328, which is incorporated by reference in its entirety herein;

15 (iii) sympathetic ganglia assay as described in Barde et al., 1982, EMBO J. 1:549-553, which is incorporated by reference in its entirety herein;

20 (iv) spinal cord neurons. Briefly, spinal cords may be removed aseptically from a test animal, severed caudal to the bulb, and freed of sensory ganglia and meninges. The cord may then be subdivided into ventral and mediodorsal segments for separate cultures, and the tissues minced into small pieces and dissociated by trituration  
25 through a Pasteur pipet in 50 percent DMEM (Gibco) and 50 percent Ham's nutrient mixture F12 (Gibco) supplemented with 33 mM glucose, 2 mM glutamine, 15 mM NaHCO<sub>3</sub>, 10 mM HEPES, 25 µg/ml insulin, 100 µg/ml transferrin, 60 µM putrescine, 20 nM  
30 progesterone, 30 nM Na selenite, 0.5 µg/ml penicillin G, 0.5 µg/ml streptomycin, and 2.5 µg/ml bovine serum albumin. Trituration may then be repeated twice and supernatants  
35 may be pooled and filtered through a 40 µm



Tetko filter. Dissociated ventral cells may then be plated in on poly-D-lysine coated (10  $\mu$ g/ml) culture dish at a density of 0.5 million cells per 35 mm dish. Dissociated mediodorsal cells may be plated at a density of 1.5 million cells per 35 mm dish coated with poly-D-lysine (10  $\mu$ g/ml), poly-L-ornithine (10 $\mu$ g/ml) or poly-L-ornithine plus laminin (5 $\mu$ g/ml).

(v) basal forebrain cholinergic neuron assays (see PCT Publication WO 91/03568, published March 21, 1991);

(vi) ventral mesencephalic dopaminergic neuron assay (see PCT Publication WO 91/03568, published March 21, 1991); and

(vii) PC12 cell assays.

## 6. EXAMPLE: CONSTRUCTION AND EXPRESSION OF THE PREPRO-NGF/MATURE-BDNF CHIMERA

### 6.1. CONSTRUCTION OF CHIMERIC NUCLEIC ACID MOLECULES USING POLYMERASE CHAIN REACTION

A polymerase chain reaction cloning was utilized (PCR; Saiki et al., 1985, Science 230: 1350-1354) to construct a prepro NGF/mature BDNF chimera consisting of the long prepro form of mouse NGF fused to the mature human BDNF sequence.

To accomplish this, two PCR primers were synthesized. The 5' primer (5'- CTC-GTC-GAC-AGC-CGG-CAC-TCT-GAC-CCT-GCG-CGC-CGA-3') [SEQ ID NO:17] encoded the first 7 amino acids of BDNF and included two unique restriction sites, NaeI and BssH2 which were generated by modifying codon usage. The 3' PCR primer was a 3' pCDM8 oligo corresponding to a region

downstream from the polylinker sequence at the 3' end of the BDNF sequence in pC8hB (5'-CAA-AGA-TCC-TCT-AGA-GTC-G-(C)-3') [SEQ ID NO:18]. The polylinker contains a NotI restriction site. These two primers were used in PCR with pC8hB (hBDNF in pCDM8) DNA as template. 5 micrograms of pC8hB was used with 500 ng of each primer for 5 PCR cycles. The PCR product was digested with both NaeI and NotI simultaneously and a 365 bp digestion product was isolated by gel electrophoresis. 10 The preparation of the vector was carried out by digesting pC81mN (long mouse NGF in pCDM8) with both Eco47 and NotI and isolating the 4.6 kb vector fragment by gel electrophoresis. The 365 bp fragment was ligated into the Eco47/NotI sites of pC81mN. This 15 ligation resulted in a direct in frame fusion of the mouse NGF prepro region with the mature BDNF coding region. Constructs were diagnostically tested by digesting with BssH2, by assessing the loss of the Eco47 site during the subcloning, and ultimately by 20 DNA sequencing.

#### 6.2. EXPRESSION OF CHIMERIC MOLECULES

CHO-DG44 cells were used to generate stable lines for the production of bioactive BDNF. CHO-DG44 25 cells (obtained from Dr. L. Chasin at Columbia University) lack both copies of the dihydrofolate reductase gene (Urlaub and Chasin, 1980, Proc. Natl. Acad. Sci. USA 77:4216-4220). Stably transfected CHO-DG44 cell lines expressing BDNF have been previously 30 described (PCT International Publication No. WO 91/03568, published March 21, 1991). These lines were generated by transfection with pC8hB DNA which encodes the human BDNF gene including the prepro region cloned into the expression vector pCDM8. CHO- 35 DG44 cells ( $1 \times 10^6$  cells/100 mm plate) were

transfected by the calcium phosphate coprecipitation method with 20  $\mu$ g of the NGF/BDNF chimera (pC81mN/B) along with 0.2  $\mu$ g of plasmid p410 which encodes a weakened dihydrofolate reductase gene (dhfr). 48 hours after transfection, the cells were passaged into selection media (Ham's F12 without hypoxanthine and thymidine containing 10% dialyzed fetal bovine serum and 1% each of penicillin and streptomycin; -HT media). -HT-resistant clones were treated as pools for amplification with methotrexate (MTX). Clones obtained with 0.05  $\mu$ M MTX were also treated as pools for further amplification at 2.5  $\mu$ M MTX. A single clone that was selected first in 0.5  $\mu$ M MTX and then in 2.5  $\mu$ M MTX (thus 2 rounds of amplification) was isolated (DGC-N/B-2.5-#23) which proved to be the highest producer of BDNF as assessed by both bioactivity and metabolic labeling. Bioactivity was assessed by scoring neurite outgrowth of embryonic (E8) chick dorsal root ganglia (DRG) (Maisonpierre et al., 1990, Science 247:1446-1451).

7. EXAMPLE: COMPARISON OF PROCESSING EFFICIENCY BETWEEN HOMOLOGOUS PREPRO BDNF AND PREPRO NGF/BDNF CHIMERA

Experiments were performed to directly compare the processing and expression of preproBDNF with the preproNGF/BDNF chimera in CHO cells.

7.1. METABOLIC LABELING

CHO-DG44 cell lines stably transfected with either pC8hB or pC81mN/B and amplified with 2.5  $\mu$ M methotrexate were compared by metabolic labeling (Figure 1). For this labeling experiment, CHO-DG44 cells expressing BDNF from either the short prepro BDNF construct (cell line = DGZ1000-B-3-2.5) or the long mouse preproNGF/BDNF chimeric construct (cell

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line = DGC-N/B-2.5-#23) were seeded at equal densities (2 x 10<sup>5</sup> cells/well in 6-well plate) 24 hours prior to labeling. The cells were then labeled with both <sup>35</sup>S-cysteine and <sup>35</sup>S-methionine for 4 hours under serum-free conditions. 30 µl aliquots of labeled cell supernatants were resolved by SDS polyacrylamide gel electrophoresis (15% gel) and labeled proteins were transferred to nylon membranes and visualized by autoradiography. As observed in Figure 1, CHO-DG44 cells stably transfected with the human NGF gene in the expression vector pCDM8 expressed the mature form of NGF migrating at a molecular weight of approximately 12,300 (Figure 1, lane 2). Wild type CHO-DG44 cells as control are shown in lane 1.

Unprocessed proBDNF (31 kD), the pro-portion of the processed proBDNF precursor (16 kD) and the mature form (14 kD) of the short preproBDNF protein were detected in the stably transfected cell line DGZ1000-B-3-2.5 (obtained after similar MTX selection and amplification as used for cell line DGC-N/B-2.5-#23) (Figure 1, lane 3). Only the proteolytically processed mature form of BDNF (14 kD) was detected in DGC-N/B-2.5-#23, stably transfected with the long proNGF/BDNF chimeric construction (Figure 1, lane 4).

Unprocessed proNGF/BDNF was not detected in the conditioned media from this cell line. We estimate from the intensity of the labeling of the mature BDNF that cell line DGC-N/B-2.5-#23 produced about five (5) times as much mature BDNF protein per cell relative to cell line DGZ1000-B-3-2.5 made with the short proBDNF construct.

## 7.2 BIOACTIVITY

The bioactivity of BDNF produced in the two CHO cell lines described above were compared. Crude supernatants were assayed with embryonic (E8) chick dorsal root ganglia and neurite outgrowth was scored. Consistent with the metabolic labeling experiments, the cell line DGC-N/B-2.5-#23 appeared to produce approximately five (5) times as much mature BDNF relative to cell line DGZ1000-B-3-2.5. For example, maximal neurite outgrowth was achieved with 10  $\mu$ l of supernatant derived from DGC-N/B-2.5-#23 cells while 50  $\mu$ l of DGZ1000-B-3-2.5 supernatant was required to achieve maximal DRG bioactivity (Figure 2).

## 7.3. COMPARISON OF EXPRESSION OF NGF USING LONG AND SHORT NGF PREPRO REGIONS

COS cells were transfected with prepro NGF containing either the long ("lmNGF") or short ("smNGF") NGF prepro region with the mature NGF coding region. Culture supernatants were harvested 48 hours after transfection and assayed on DRG explants, along with purified NGF and a mock transfected COS cell supernatant. Results using three different concentrations of each construct, as shown in Table 1, reveal significant bioactivity of NGF expressed with either the long or short form of the prepro region.

TABLE 1  
Effect of Various COS  
Supernatants on DRG Explants

SAMPLE	DILUTION	DRG
(-) CONTROL NGF	10 ng/ml	0,0,0,0,0.5 5+,5+,5+,5+,5+
MOCK	10 $\mu$ l	0,1,1,1,1
	50 $\mu$ l	0.5,1,1,1,1.5
	100 $\mu$ l	0.5,0.5,1,1,1
	250 $\mu$ l	2,2.5,2.5,2.5,2.5
smNGF	10 $\mu$ l	2,3,3,3,3.5
	50 $\mu$ l	5,5,5,5,5
	100 $\mu$ l	5+,5+,5+,5+,5+
	250 $\mu$ l	5+,5+,5+,5+,5+
lmNGF	10 $\mu$ l	2,2,2,2,2
	50 $\mu$ l	4,4,4,4,4
	100 $\mu$ l	5,5,5,5,5
	250 $\mu$ l	5,5,5,5,5

#### 7.4. CONCLUSIONS

We conclude from these studies that the long pro portion of NGF is better suited for the processing of BDNF in CHO cells than the short pro portion of BDNF. The advantages of the chimeric proNGF/mature BDNF gene construct, therefore, is that it allows for higher expression levels of BDNF on a per cell basis in mammalian cells. Additionally, it should allow for better purification schemes for BDNF in that contaminating unprocessed forms of BDNF are not apparent in the crude supernatants.

Additionally, use of either the long or short prepro region of NGF results in the expression of biologically active NGF. This indicates that either the long or short prepro region of NGF may be utilized in the construction of chimeric neurotrophic genes.

#### 8. EXAMPLE: CONSTRUCTION AND EXPRESSION OF THE PREPRO-NT-3/BDNF CHIMERA

##### 8.1. CONSTRUCTION OF CHIMERIC NUCLEIC ACID MOLECULES

A HindIII-XhoI DNA fragment containing the entire coding region of prepro and mature human BDNF was obtained from digestion of plasmid pC8hB with corresponding restriction enzymes. The plasmid pC8hB was derived by cloning the human BDNF coding sequences, including the entire prepro region, into the expression vector pCDM8 (discussed supra). This fragment was ligated to pDSR $\alpha$ 2 (see published European patent application 90305433.6 EPO Publication No. 0398753A2, incorporated herein by reference in its entirety. The plasmid pDSR $\alpha$ 2 had been previously digested to make available the cloning sites 5'-HindIII and 3'-SalI for ligation of the human BDNF

containing fragment. The resulting plasmid was designated pDSR $\alpha$ 2(BDNF).

For generating a chimeric plasmid with a prepro NT-3 sequence and a mature BDNF sequence, three DNA fragments were prepared as follows and then ligated in a specific orientation. An approximately 400-bp 5'-HindIII/3'-NarI DNA fragment containing all of the prepro human BDNF sequence was deleted by restriction enzyme digestion from the expression plasmid pDSR $\alpha$ 2(BDNF) described above. A DNA fragment recovered from this digestion contained the entire expression vector pDSR $\alpha$ 2 and the mature human BDNF sequence, bordered by the 5'-HindIII and a 3'-NarI sites (labeled DNA fragment No. 1). An approximately 300-base pair 5'-HindIII/3'-SacII DNA fragment containing the prepro region of human NT-3 was obtained through digestion of plasmid pC8hN3 with corresponding restriction enzymes. Coding sequences corresponding to 35 amino acid residues of the prepro NT-3 region were deleted downstream of the SacII site as a consequence of the digestion. The plasmid pC8hN3 was derived by cloning the human NT-3 coding sequences, including the entire prepro region, into the expression vector pCDM8. The 300-base pair 5'-HindIII/3'-SacII fragment was labeled DNA fragment No. 2. Finally, DNA fragment No. 3 was prepared, which was an oligonucleotide linker synthesized to regenerate the aforementioned missing 35 amino acid residues (Figure 6 and SEQ ID NO:15-17). The linker also contained the half sites of the 5'-SacII and 3'-NarI restriction sites to promote ligation to DNA fragments Nos. 1 and 2 disclosed supra. This ligation resulted in the expression vector pDSR $\alpha$ 2(NT-3/BDNF), in which the prepro region of NT-3 (fragment No. 2) is joined with mature BDNF (fragment No. 1) by the



oligonucleotide linker (fragment No. 3; Figure 6 and SEQ ID NO:15).

## 8.2. EXPRESSION AND CHARACTERIZATION OF NT-3/BDNF CHIMERA IN CHO CELLS

5 CHO-D(-) cells (ATCC accession number CCL 61) were used to generate stable lines for the production of bioactive BDNF. CHO-D(-) cells are defective in the gene encoding dihydrofolate reductase and are maintained in the medium of Dulbecco's  
10 modified Eagle media (D-MEM), supplemented with MEM nonessential amino acids, 1% each of penicillin and streptomycin, 10% fetal bovine serum, hypoxanthine and thymidine. CHO-D(-) cells ( $0.8 \times 10^6$ /60 mm plate) were  
15 transfected by the calcium phosphate coprecipitation method, using 2.5  $\mu$ g of the NT-3/BDNF chimeric construction [pDSR $\alpha$ 2(NT-3/BDNF)] previously linearized by digestion with restriction enzyme PvuI. This vector (pDSR $\alpha$ 2) encodes a mouse dihydrofolate  
20 reductase minigene (dhfr) which, when expressed, enables the transfected CHO-D(-) to overcome the deficiency of the dhfr gene and become capable of growing in the absence of the nucleotides hypoxanthine and thymidine. Parental CHO-D(-) cells or cells not  
25 successfully transfected by the vector pDSR $\alpha$ 2 will not survive in the selection media, which has the composition of the maintenance media described above except that fetal bovine serum is substituted with dialyzed fetal bovine serum and hypoxanthine and  
30 thymidine are omitted. The cells were trypsinized and seeded 48 hours after transfection at  $1 \times 10^5$  cells/100 mm plate in selection media. Individual colonies were picked two weeks later using cloning cylinders. Each clone was then expanded to 100 mm plates. When the  
35 cultures reached confluency, the original serum-

containing media were aspirated and replaced with 3 ml of serum free media. The conditioned media (CM) were collected and 50  $\mu$ l each was loaded on a 15% SDS-polyacrylamide gel and subjected to gel electrophoresis. Western blotting of the gel was performed with rabbit antiserum specific for mature BDNF. As shown in Figure 7, all clones expressing the original BDNF from pDSR $\alpha$ 2(BDNF) secreted multiple forms of unprocessed BDNF, in addition to the mature, processed BDNF. The ratio of unprocessed forms to processed form was about 2:1. In contrast, all of the clones expressing chimeric NT-3/BDNF from pDSR $\alpha$ 2(BDNF) secreted only the fully processed, mature form of BDNF with no detectable partially processed precursors.

One liter of serum-free conditioned media from one of the chimeric NT-3/BDNF clones was subjected to purification by passage through an S-Sepharose column followed by a Sephacryl S-200 size exclusion column. SDS-PAGE analysis and amino acid sequence determination showed that a homogeneous protein with a molecular weight of 14 kd (as predicted for mature human BDNF) was obtained, with a unique N-terminal sequence in agreement with the N-terminal sequence of mature human BDNF. Furthermore, the purified BDNF was demonstrated by the chick dorsal root ganglia assay (described for the NGF/BDNF chimera, supra) to possess full biological activity.

### 8.3. EXPRESSION IN COS CELLS AND BIOACTIVITY OF THE NT-3/BDNF CHIMERA

COS-7 cells (ATCC accession number CRL 1651) were used as a transient expression system to test the production of bioactive BDNF. COS-7 cells are routinely maintained in D-MEM with 10% fetal bovine serum and 1% penicillin and streptomycin antibiotics.

COS-7 cells ( $5 \times 10^6$  cells/ml) were transfected by electroporation at 1600 volts for 0.4 msec with, individually, 20  $\mu$ g each of pDSR $\alpha$ 2, pDSR $\alpha$ 2(BDNF) and pDSR $\alpha$ 2(NT-3/BDNF). Transfected COS-7 cells were  
5 plated at  $2 \times 10^6$  cells/60 mm plate. Conditioned medium accumulated between 24 and 72 hours post transfection was collected. Bioactivity was assessed by scoring neurite outgrowth of embryonic (E8) chick dorsal root ganglia (as with the NGF/BDNF chimera).  
10 As shown in Table 2, the clonal isolates CI 1 and CI 20 of chimeric pDSR $\alpha$ 2(NT-3/BDNF) were approximately 5 times more active than mature BDNF expressed from pDSR $\alpha$ 2(BDNF), the latter containing the unaltered prepro region of BDNF.

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TABLE 2  
Chick DRG Explant Assay  
of Conditional Media from COS Cells  
Transfected with Plasmid DNA

5	DNA Source	Volume of Medium Tested $\mu$ L	Score of Neurite Outgrowth
	pDSR $\alpha$ 2	10 50	0,0,0,0,0 0,0,0,0.5,0.5
10	pDSR $\alpha$ 2 (BDNF)	10 50	0,0.5,0.5,0.5,0.5 1,1,1,1.5,1.5
	pDSR $\alpha$ 2 (NT-3/BDNF), CI 2	10 50	1,1,1.5,1.5,1.5 2.5,2.5,2.5,2,2
15	pDSR $\alpha$ 2 (NT-3/BDNF), CI 20	10 50	1,1,2,2,2 2.5,2.5,2.5,2.5,2.5

#### 8.4 CONCLUSIONS

These studies demonstrate that the substitution of the prepro region of BDNF with the NT-3 prepro region facilitates the proteolytic processing of the prepro region and significantly increases the net yield of mature BDNF. Further, the reconstituted cleavage site between the prepro NT-3 and mature BDNF DNA sequences was recognized accurately by the host cell without any alteration at the NH<sub>2</sub>-terminus of the mature, processed BDNF. As with the chimeric NGF/BDNF gene construct, the chimeric NT-3/BDNF gene construct results for higher levels of processed BDNF on a per cell basis in mammalian cells, and it should also allow for better purification schemes by elimination or minimization of contaminating unprocessed forms.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in

addition to those described herein will become  
apparent to those skilled in the art from the  
foregoing description and accompanying figures. Such  
modifications are intended to fall within the scope of  
5 the appended claims.

Various publications and patent applications  
are cited herein, the disclosures of which are  
incorporated by reference in their entireties.

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WHAT IS CLAIMED IS:

1. A chimeric prepro protein or prepro  
5 peptide comprising (a) a prepro region of a first  
neurotrophin; and (b) an amino acid sequence  
substantially equivalent to the mature form of a  
second neurotrophin, in which the first and second  
neurotrophins are different.
- 10 2. A chimeric prepro protein or prepro  
peptide comprising (a) prepro region of a first  
neurotrophin; and (b) a biologically active amino acid  
sequence substantially equivalent to a portion of the  
15 mature form of a second neurotrophin, in which the  
first and second neurotrophins are different.
3. The chimeric prepro protein of claim 1  
in which the first and second neurotrophins are  
20 selected from the group consisting of nerve growth  
factor, brain-derived neurotrophic factor, and  
neurotrophin-3.
4. The chimeric prepro protein of claim 2  
25 in which the first and second neurotrophins are  
selected from the group consisting of nerve growth  
factor, brain-derived neurotrophic factor, and  
neurotrophin-3.
- 30 5. The chimeric prepro protein or prepro  
peptide of claim 1 in which the prepro region is the  
long prepro region of nerve growth factor.

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6. The chimeric prepro protein or prepro peptide of claim 2 in which the prepro region is the long prepro region of nerve growth factor.

7. The chimeric prepro protein or prepro peptide of claim 1 in which the prepro region is the short prepro region of nerve growth factor.

8. The chimeric prepro protein or prepro peptide of claim 2 in which the prepro region is the short prepro region of nerve growth factor.

9. The chimeric prepro protein or prepro peptide of claim 1 in which the first neurotrophin is neurotrophin-3.

10. The chimeric prepro protein or prepro peptide of claim 2 in which the first neurotrophin is neurotrophin-3.

11. The chimeric prepro protein or prepro peptide of claim 1 in which the first neurotrophin is brain-derived neurotrophic factor.

12. The chimeric prepro protein or prepro peptide of claim 2 in which the first neurotrophin is brain-derived neurotrophic factor.

13. The chimeric prepro protein or prepro peptide of claim 5 or 6 in which the second neurotrophin is brain-derived neurotrophic factor.

14. The chimeric prepro protein or prepro peptide of claim 7 or 8 in which the second neurotrophin is brain-derived neurotrophic factor.

15. The chimeric prepro protein or prepro peptide of claim 9 or 10 in which the second neurotrophin is brain-derived neurotrophic factor.

5 16. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 1.

10 17. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 2.

15 18. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 3.

20 19. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 4.

20. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 5.

25 21. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 6.

30 22. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 7.

35 23. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 8.



24. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 9.

5           25. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 10.

10           26. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 11.

15           27. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 12.

20           28. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 13.

          29. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 14.

25           30. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 15.

30           31. The nucleic acid molecule of claim 16 or 17, which is a vector.

          32. The nucleic acid molecule of claim 18 or 19, which is a vector.

33. A recombinant cell containing the vector of claim 31.

34. A recombinant cell containing the  
5 vector of claim 32.

35. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 1, under conditions  
10 such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the mature form of the second neurotrophin.

36. A method of producing a neurotrophin  
15 comprising growing a recombinant cell containing the nucleic acid molecule of claim 2, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the portion of the mature form of the second neurotrophin.  
20

37. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 3, under conditions  
25 such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the mature form of the second neurotrophin.

38. A method of producing a neurotrophin comprising growing a recombinant cell containing the  
30 nucleic acid molecule of claim 4, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the portion of the mature form of the second neurotrophin.

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39. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 5, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the mature form of the second neurotrophin.

40. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 6, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the portion of the mature form of the second neurotrophin.

41. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 7, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the mature form of the second neurotrophin.

42. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 8, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the portion of the mature form of the second neurotrophin.

43. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 9, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the mature form of the second neurotrophin.

44. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 10, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the portion of the mature form of the second neurotrophin.

45. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 11, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the mature form of the second neurotrophin.

46. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 12, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the portion of the mature form of the second neurotrophin.

47. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 13, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the mature form of the second neurotrophin.

48. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 14, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the mature form of the second neurotrophin.

49. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 15, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the mature form of the second neurotrophin.

50. The method according to claim 35 or 36 in which the produced mature form or portion thereof of the second neurotrophin is capable of exhibiting neurotrophic activity.

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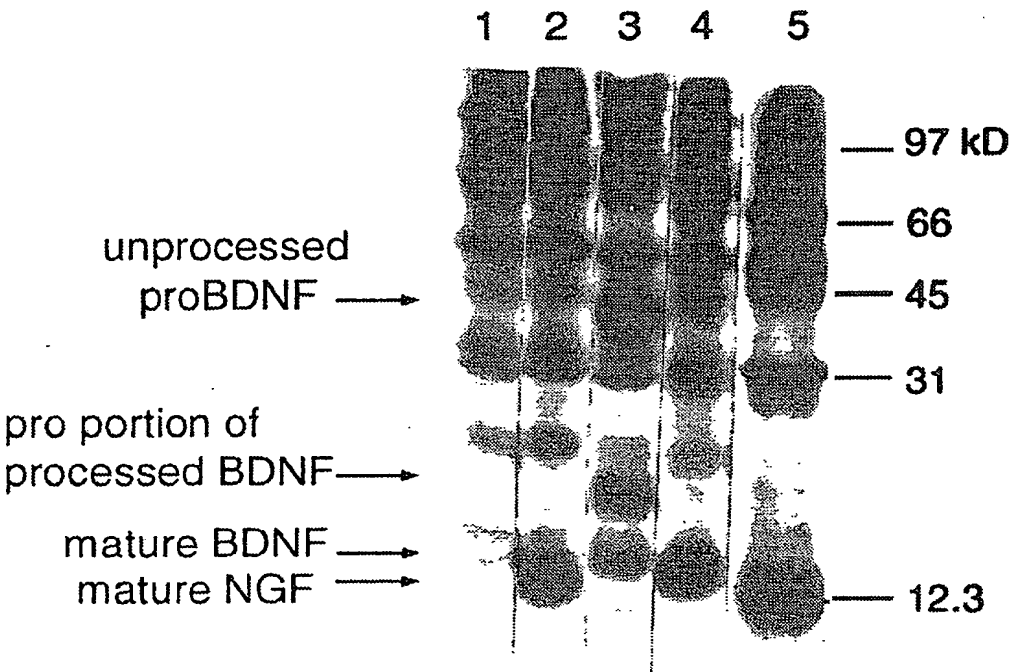
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FIG. I



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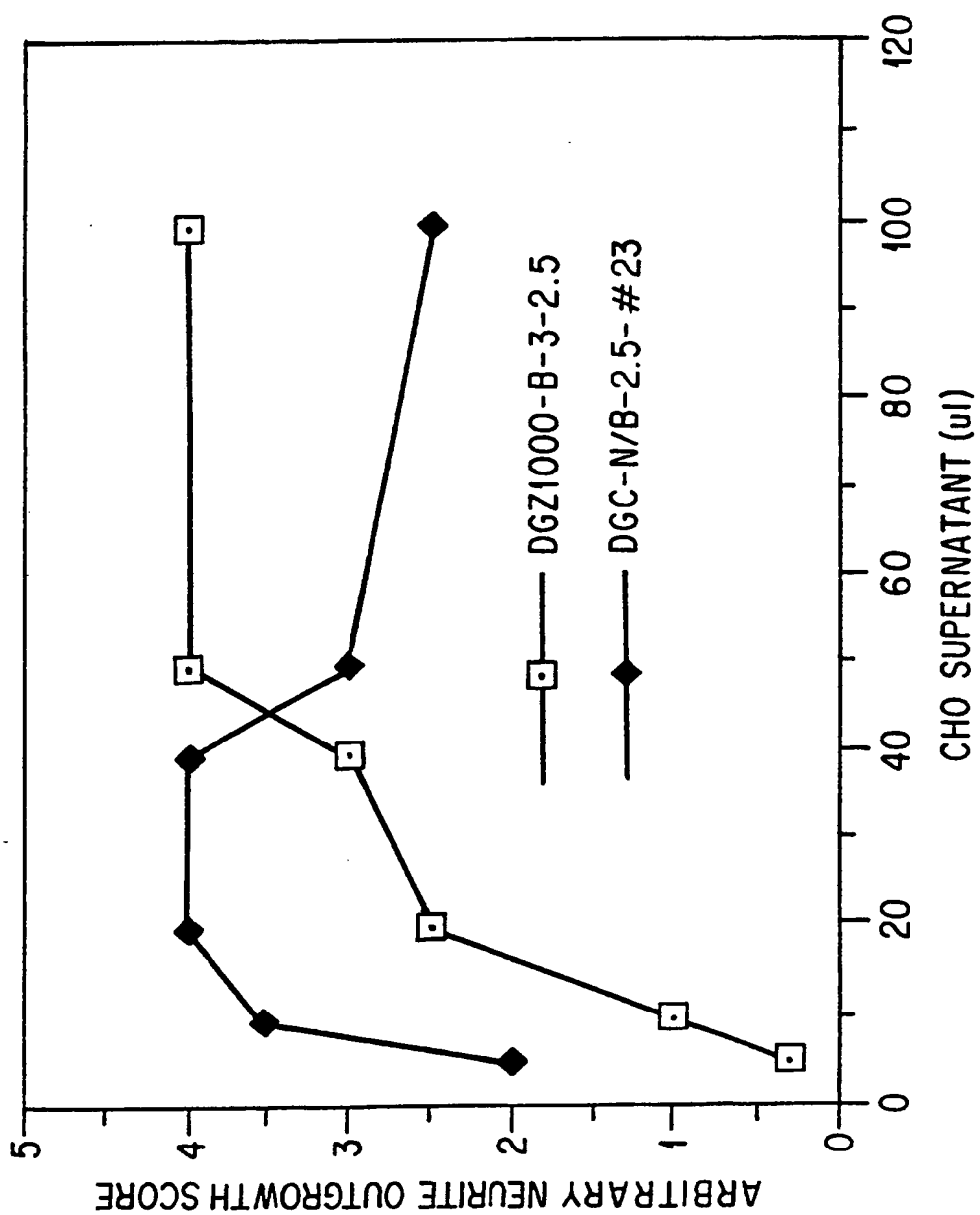


FIG. 2

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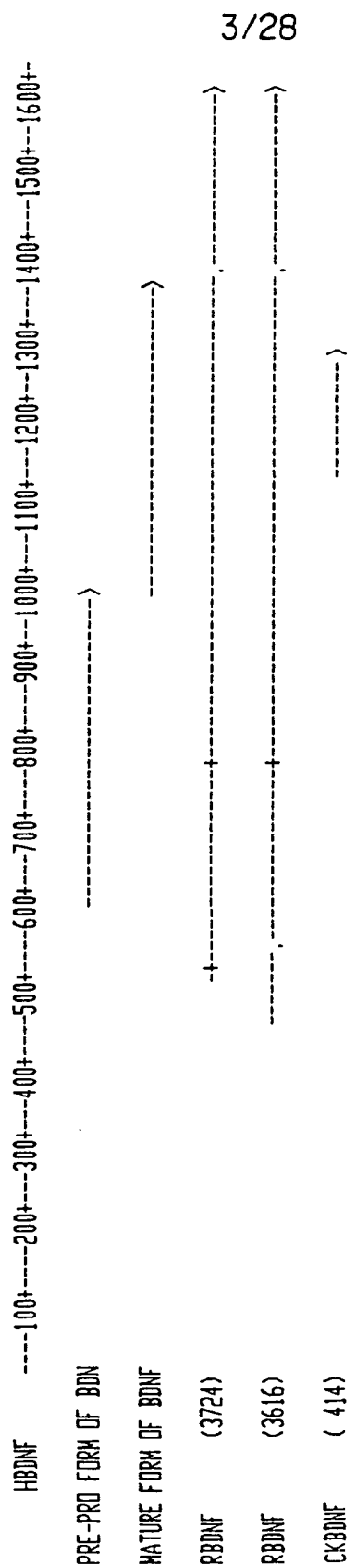


FIG. 3



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	10	20	30	40	50	60	70	80	90	100	110	120	
HUMAN	AAGCTTGATATCGAATTCGGAAATCCGTTCCCAACTGCTGTTTATTGTGCTATTTCAGCTAGAGATCAGAGAGGCCATCAGAGCCCTCAGGCGACTGCTGTTCTGTGCACACATTC TTCGAACATATAGCTTAAGGCTTAAGGCAAGGGTTGACGACAAATAACACGATAAGTAGCGATCTGTAGTGATCGATCTTCCGGGTAGTCTGGGGAGTCCGGTGACGACAGGACAGTGTGTAAG												
	130	140	150	160	170	180	190	200	210	220	230	240	250
HUMAN	CTGCAAGGACCATGTTGCTAACTTGAAGAAATTAATTAATTAACACTTGCAGTTGTTGCTTAGTAACATTTATGATTTTGTGTTCTCGTGACAGCATGACGAGATCATTAAGAAATTAACCTTA GACGTTTCCCTGGTACACAGATTGAACTTTTAAATGATAATTAATGTGACGTCACACAGCAATCATTTGTAAATACTAAACACAAAGGACACTGTCGTACTGCTCTAGTAAATTTTAAATTTGAAAT												
	260	270	280	290	300	310	320	330	340	350	360	370	380
HUMAN	CAAAGCTGCTAAAGTGGGAGAGGAGAACTTGAAGCCACAATTTTGCACCTTGTAGAGCCATCTAATCTCAGGTTATATGCTAGATCTTGGGGCAACACTGCAATGCTCTGTTTATATATAA GTTTCGACGATTCACCCCTCTCTCTTGAACCTTCGGTGTAAAGAGTGACGAAATCTTCGGTAGATTAGAGTCCAAATATACGATCTAGAACCCCGTTTGTGACGTACAGAGACCAATAATAATTT												
	390	400	410	420	430	440	450	460	470	480	490	500	510
HUMAN	CCACATACAGCAGCTACTGACACTGATTTGTGCTGGTGCAGCTGGAGTTATCACCAGACATAAAAAACCTTGACCCCTGCAGATGGCTTGGAAATTACAATCAGATGGGCCACATGGCATCCCGG GGTGTATGTCGTGTGATGACTGTGACTAAACACAGACAGCAGCTCGACCTCAATAGTGGTTCGTATTTTGGAACTGGGACGCTTACCGGACCTTAAATGTTAGTCTACCCGGGTGTAACCGGTAGGGCC												

HBDF

C

RAT 4  
PIG 39  
...CA...GGG...G.G...AAAATA.T.GA.T.TG...G...-----G.A.T.T...-C>  
...A...T...>

FIG. 3A

[illegible]

**FIG. 3B**

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[illegible]

RAT	129	... .. G ... .. AC ... .. C AA ... .. C ... .. C ... ..
PIG	209	... .. C ... .. C ... .. C A ... .. C A ... .. C A ... ..

	JHBDNF										
	730	740	750	760	770	780	790	800	810	820	
HUMAN	AAT GGG CCC AAG GCA GGT TCA ACA GGC TTG ACA TCA TTG GCT GAC ACT TTC GAA CAC GTG ATA CAA GAG CTG TTG GAT GAG CAC CAG AAA GTT CGG	TTA CCC GGG TTC CGT CCA AGT TCT CCG AAC TGT AGT AAC CGA CTG TGA AAG CTT GTG CAC TAT CTT CTC GAC AAC CTA CTC CTG GTC TTT CAA GGC	Asn Gly Pro Lys Ala Gly Ser Arg Gly Leu Thr Ser Leu Ala Asp Thr Phe Glu His Val Ile Glu Glu Leu Asp Glu Asp Gln Lys Val Arg								

RAT	225	... ..G.	... ..G	....I	C..	.C	C..	....T	.G	....C	....C..	....G	.....
<div style="text-align: center;">CGACGA ^</div>													

ATCGTGGTCATCGTC  
^  
P[6] 305 ... .. C.. .G .G ... .I ... ..C ..G ... ..C

**FIG. 3C**

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[illegible]

**FIG. 3D**

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	HBDNF										
	920	930	940	950	960	970	980	990	1000	1010	
HUMAN	GAG GAA TAC AAA AAT TAC CTA GAT GCT GCA AAC ATG TCC ATG AGG GTC CGG CCG CAC TCT GAC CCT GCC CCG CGA GGG GAG CTG AGC GTG TGT GAC	CTC CTT ATG TTT TTA ATG GAT CTA CGA CGT TTG TAC AGG TAC TCC CAG GCC GCG GGT AGA CTG GGA CCG GCG GCT CCC CTC GAC TCG CAC ACA CTG	Glu Glu Tyr Lys Asn Tyr Leu Asp Ala Ala Asn Met Ser Met Arg Val Arg Arg	His Ser Asp Pro Ala Arg Arg Gly Glu Leu Ser Val Cys Asp							
	b b b b PRE-PRO FORM OF BDNF a a a a a a b b b b MATURE FORM OF BDNF b b b b										
RAT 423	...	...	...	...	...	...	...	...	...	...	
PIG 512	...	...	...	...	...	...	...	...	...	...	
	1020	1030	1040	1050	1060	1070	1080	1090	1100	1110	
HUMAN	AGT ATT AGT GAG TGG GTA ACG GCG GCA GAC AAA AAG ACT GCA GTG GAC ATG TCG GGC GGG ACG GTC ACA GTC CTT GAA AAG GTC CCT GTA TCA AAA	TCA TAA TCA CTC ACC CAT TGC CGC CGT CTG TTT TTC TGA CGT CAC CTG TAC AGC CCG CCC TGC CAG TGT CAG GAA CTT TTC CAG GGA CAT AGT TTT	Ser Ile Ser Glu Trp Val Thr Ala Ala Asp Lys Lys Thr Ala Val Asp Met Ser Gly Gly Thr Val Thr Val Leu Glu Lys Val Pro Val Ser Lys	b b b b b b b b b b MATURE FORM OF BDNF b b b b b b b b b b							
RAT 519	...	...	...	...	...	...	...	...	...	...	
PIG 608	...	...	...	...	...	...	...	...	...	...	

FIG. 3E

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**FIG. 3F**



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[illegible]

RAT 922 ..G.....A.....A.....I.....

III ^

.....PIG 1012.....I.....AA.....

	1560	1570	1580	1590	1600	1610	HBDNF

HUMAN  
ACATTCTTGATAATGTTGTGGTTGTTGCCGTGGCCAGAACTGAAACGGAATTCCTGCAG  
TGTAGGAACTATTACACACCAACACACGCAACGGTCTTGACTTTGGCTTTAAGGACGTC

RAT 1050 .....C.....T.....AA..

PIG 1140 .....C.....

**FIG. 3H**



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AGCGCATCGA GTGACTTTGG AGCTGGCCTT ATATTGGAT CTCCCGGGCA GCTTTTGGGA

AACTCCTAGT GAAC ATG CTG TGC CTC AAG CCA GTG AAA TTA GGC TCC CTG

Met Leu Cys Leu Lys Pro Val Lys Leu Gly Ser Leu

-187

-180

GAG GTG GGA CAC GGG CAG CAT GGT GGA GTT TTG GCC AGT GGT CGT GCA  
Glu Val Gly His Gly Gln His Gly Gly Val Leu Ala Ser Gly Arg Ala

-170

-160

GTC CAA GGG GCT GGA TGG CAT GCT GGA CCC AAG CTC AGC TCA GCG TCC  
Val Gln Gly Ala Gly Trp His Ala Gly Pro Lys Leu Ser Ser Ala Ser

-150

GGA CCC AAT AAC AGT TTT ACC AAG GGA GCA GCT TTC TAT CCT GGC CAC  
Gly Pro Asn Asn Ser Phe Thr Lys Gly Ala Ala Phe Tyr Pro Gly His

-140

-130

ACT GAG GTG CAT AGC GTA ATG TCC ATG TTG TTC TAC ACT CTG ATC ACA  
Thr Glu Val His Ser Val Met Ser Met Leu Phe Tyr Thr Leu Ile Thr

-120

FIG. 4

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GCT TTT CTG ATC GGC ATA CAG GCG GAA CCA CAC TCA GAG AGC AAT GTC  
Ala Phe Leu Ile Gly Ile Gln Ala Glu Pro His Ser Glu Ser Asn Val

-110

-100

CCT GCA GGA CAC ACC ATC CCC CAA GTC CAC TGG ACT AAA CTT CAG CAT  
Pro Ala Gly His Thr Ile Pro Gln Val His Trp Thr Lys Leu Gln His

-90

-80

TCC CTT GAC ACT GCC CTT CGC AGA GCC CGC AGC GCC CCG GCA GCG GCG  
Ser Leu Asp Thr Ala Leu Arg Arg Ala Arg Ser Ala Pro Ala Ala Ala

-70

ATA GCT GCA CGC GTG GCG GGG CAG ACC CGC AAC ATT ACT GTG GAC CCC  
Ile Ala Ala Arg Val Ala Ala Gly Gln Thr Arg Asn Ile Thr Val Asp Pro

-60

-50

AGG CTG TTT AAA AAG CGG CGA CTC CGT TCA CCC CGT GTG CTG TTT AGC  
Arg Leu Phe Lys Lys Arg Arg Arg Leu Arg Ser Pro Arg Val Leu Phe Ser

-40

FIG. 4A

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ACC CAG CCT CCC CGT GAA GCT GCA GAC ACT CAG GAT CTG GAC TTC GAG  
 Thr Gln Pro Pro Arg Glu Ala Ala Asp Thr Gln Asp Leu Asp Phe Glu  
 -30 -20

GTC GGT GGT GCT GCC CCC TTC AAC AGG ACT CAC AGG AGC AAG CCG TCA  
 Val Gly Gly Ala Ala Pro Phe Asn Arg Thr His Arg Ser Lys Arg Ser  
 -10 1

TCA TCC CAT CCC ATC TTC CAC AGG GGC GAA TTC TCG GTG TGT GAC AGT  
 Ser Ser His Pro Ile Phe His Arg Gly Glu Phe Ser Val Cys Asp Ser  
 10

GTC AGC GTG TGG GTT GGG GAT AAG ACC ACC GCC ACA GAC ATC AAG GGC  
 Val Ser Val Trp Val Gly Asp Lys Thr Thr Ala Thr Asp Ile Lys Gly  
 20 30

AAG GAG GTG ATG GTG TTG GGA GAG GTG AAC ATT AAC AAC AGT GTA TTC  
 Lys Glu Val Met Val Leu Leu Gly Glu Val Asn Ile Asn Asn Ser Val Phe  
 40

AAA CAG TAC TTT TTT GAG ACC AAG TGC CGG GAC CCA AAT CCC GTT GAC  
 Lys Gln Tyr Phe Phe Glu Thr Lys Cys Arg Asp Pro Asn Pro Val Asp  
 50 60

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FIG. 4B

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AGC GGG TGC CGG GGC ATT GAC TCA AAG CAC TGG AAC TCA TAT TGT ACC  
Ser Gly Cys Arg Gly Ile Asp Ser Lys His Trp Asn Ser Tyr Cys Thr

70

80

ACG ACT CAC ACC TTT GTC AAG GCG CTG ACC GTG GAT GGC AAG CAG GCT  
Thr Thr His Thr Phe Val Lys Ala Leu Thr Met Asp Gly Lys Gln Ala

90

GCC TGG CGG TTT ATC CGG ATA GAT ACG GCC TGT GTG TGT GTG CTC AGC  
Ala Trp Arg Phe Ile Arg Ile Asp Thr Ala Cys Val Cys Val Leu Ser

100

110

AGG AAG GCT GTG AGA AGA GCC TGACCTGCCG ACACGCTCCC TCCCCCTGCC  
Arg Lys Ala Val Arg Arg Ala

120

CCTTCTACAC TCTCCTGGGC CCCTCCCTAC CTCAACCTGT AAATTATTTT AAATTATAAG

GACTGCATGG TAATTATAG TTTATACAGT TTAAAGAAT CATTATTTAT TAAATTTTGG

GAAGCATCCT GTGTGCTGA

FIG. 4C

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Sequence Range: 9 to 1142 | r/NT-3gene | R  
 Sequence Range: 33 to 1057 | h/NT-3gene | H

10 \* 20 \* 30 \* 40 \* 50 \*  
 GA TTCCATAA TGA CCC AGA CTC TTC CAG TCA GAT ATT AAC ACT TGT GTT  
 CT AAGGTATT ACT GGG TCT GAG AAG GTC AGT CTA TAA TTG TGA ACA CAA  
 End Pro Arg Leu Phe Gln Ser Asp Ile Asn Thr Cys Val>

R

40 \* 50 \* 60 \* 70 \* 80 \*  
 TGCCAGAA TAA CAC AGA CTC AGC TGC CAG AGC CTG CTC TTA ACA CCT GTG  
 ACGGTCTT ATT GTG TCT GAG TCG ACG GTC TCG GAC GAG AAT TGT GGA CAC  
 End His Arg Leu Ser Cys Gln Ser Leu Leu Thr Pro Val>

H

60 \* 70 \* 80 \* 90 \* 100 \*  
 TCC TTC TTT CAG ATC TTA CAG GTG AAC AAG GTG ATG TCC ATC TTG TTT  
 AGG AAG AAA GTC TAG AAT GTC CAC TTG TTC CAC TAC AGG TAG AAC AAA  
 Ser Phe Phe Gln Ile Leu Gln Val Asn Lys Val

R

Met Ser Ile Leu Phe>  
 PREPRO >>

90 \* 100 \* 110 \* 120 \* 130 \*  
 TTT CCT TTT CAG ATC TTA CAG GTG AAC AAG GTG ATG TCC ATC TTG TTT  
 AAA GGA AAA GTC TAG AAT GTC CAC TTG TTC CAC TAC AGG TAG AAC AAA  
 Phe Pro Phe Gln Ile Leu Gln Val Asn Lys Val

H

Met Ser Ile Leu Phe>  
 PREPRO >>

FIG. 5

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110	*	120	*	130	*	140	*	150	*	
TAT GTG ATA TTT CTT GCT TAT CTC CGT GGC ATC CAA GGC AAC AAC ATG										R
ATA CAC TAT AAA GAA CGA ATA GAG GCA CCG TAG GTT CCG TTG TTG TAC										
Tyr Val Ile Phe Leu Ala Tyr Leu Arg Gly Ile Gln Gly Asn Asn Met>										
140	*	150	*	160	*	170	*			
TAT GTG ATA TTT CTC GCT TAT CTC CGT GGC ATC CAA GGT AAC AAC ATG										H
ATA CAC TAT AAA GAG CGA ATA GAG GCA CCG TAG GTT CCA TTG TTG TAC										
Tyr Val Ile Phe Leu Ala Tyr Leu Arg Gly Ile Gln Gly Asn Asn Met>										
160	*	170	*	180	*	190	*	200	*	
GAT CAA AGG AGT TTG CCA GAA GAC TCT CTC AAT TCC CTC ATT ATC AAG										R
CTA GTT TCC TCA AAC GGT CTT CTG AGA GAG TTA AGG GAG TAA TAG TTC										
Asp Gln Arg Ser Leu Pro Glu Asp Ser Leu Asn Ser Leu Ile Ile Lys>										
180	*	190	*	200	*	210	*	220	*	
GAT CAA AGG AGT TTG CCA GAA GAC TCG CTC AAT TCC CTC ATT ATT AAG										H
CTA GTT TCC TCA AAC GGT CTT CTG AGC GAG TTA AGG GAG TAA TAA TTC										
Asp Gln Arg Ser Leu Pro Glu Asp Ser Leu Asn Ser Leu Ile Ile Lys>										

FIG. 5A

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210 \*      220 \*      230 \*      240 \*  
 TTG ATC CAG GCG GAT ATC TTG AAA AAC AAG CTC TCC AAG CAG ATG GTA  
 AAC TAG GTC CGC CTA TAG AAC TTT TTG TTC GAG AGG TTC GTC TAC CAT      R  
  
 Leu Ile Gln Ala Asp Ile Leu Lys Asn Lys Leu Ser Lys Gln Met Val>  
  
 230 \*      240 \*      250 \*      260 \*      270 \*  
 CTG ATC CAG GCA GAT ATT TTG AAA AAC AAG CTC TCC AAG CAG ATG GTG  
 GAC TAG GTC CGT CTA TAA AAC TTT TTG TTC GAG AGG TTC GTC TAC CAC      H  
  
 Leu Ile Gln Ala Asp Ile Leu Lys Asn Lys Leu Ser Lys Gln Met Val>  
  
 250 \*      260 \*      270 \*      280 \*      290 \*  
 GAT GTT AAG GAA AAT TAC CAG AGC ACC CTG CCC AAA GCA GAG GCA CCC  
 CTA CAA TTC CTT TTA ATG GTC TCG TGG GAC GGG TTT CGT CTC CGT GGG      R  
  
 Asp Val Lys Glu Asn Tyr Gln Ser Thr Leu Pro Lys Ala Glu Ala Pro>  
  
 280 \*      290 \*      300 \*      310 \*      320 \*  
 GAC GTT AAG GAA AAT TAC CAG AGC ACC CTG CCC AAA GCT GAG GCT CCC  
 CTG CAA TTC CTT TTA ATG GTC TCG TGG GAC GGG TTT CGA CTC CGA GGG      H  
  
 Asp Val Lys Glu Asn Tyr Gln Ser Thr Leu Pro Lys Ala Glu Ala Pro>

FIG. 5B

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300 \* 310 \* 320 \* 330 \* 340 \*  
 AGA GAA CCA GAG CAG GGA GAG GCC ACC AGG TCA GAA TTC CAG CCG ATG  
 TCT GTT GGT CTC GTC CCT CTC CGG TGG TCC AGT CTT AAG GTC GGC TAC

R

Arg Glu Pro Glu Gln Gly Gly Ala Thr Arg Ser Gly Phe Gln Pro Met

330 \* 340 \* 350 \* 360 \* 370 \*  
 CGA GAG CCG GAG CAG GGA GGG CCC CGC AAG TCA GCA TTC CAG CCA GTG  
 GCT CTC GGC CTC GTC CCT CCC GGG GCG TTC AGT CGT AAG GTC GGT CAC

H

Arg Glu Pro Glu Gln Gly Gly Pro Arg Lys Ser Ala Phe Gln Pro Val>

350 \* 360 \* 370 \* 380 \* 390 \*  
 ATT GCA ACA GAC ACA GAA CTA CTA CGG CAA CAG AGA CGC TAC AAT TCA  
 TAA CGT TGT CTG TGT CTT GAT GAT GCC GTT GTC TCT GCG ATG TTA AGT

R

Ile Ala Thr Asp Thr Glu Leu Leu Arg Gln Gln Arg Arg Tyr Asn Ser>

380 \* 390 \* 400 \* 410 \*  
 ATT GCA ATG GAC ACC GAA CTG CTG CGA CAA CAG AGA CGC TAC AAC TCA  
 TAA CGT TAC CTG TGG CTT GAC GAC GCT GTT GTC TCT GCG ATG TTG AGT

H

Ile Ala Met Asp Thr Glu Leu Leu Arg Gln Gln Arg Arg Tyr Asn Ser>

FIG. 5C

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400 \* 410 \* 420 \* 430 \* 440 \*  
 CCC CGG GTC CTG CTG AGT GAC AGC ACC CCT TTG GAG CCC CCT CCC TTA  
 GGG GCC CAG GAC GAC TCA CTG TCG TGG GGA AAC CTC GGG GGA GGG AAT  
 R  
 Pro Arg Val Leu Ser Asp Ser Thr Pro Leu Glu Pro Pro Pro Leu>  
 420 \* 430 \* 440 \* 450 \* 460 \*  
 CCG CGG GTC CTG CTG AGC GAC ACG ACC CCC TTG GAG CCC CCG CCC TTG  
 GGC GCC CAG GAC GAC TCG CTG TGC TGG GGG AAC CTC GGG GGC GGG AAC  
 H  
 Pro Arg Val Leu Ser Asp Thr Thr Pro Leu Glu Pro Pro Pro Leu>  
 450 \* 460 \* 470 \* 480 \*  
 TAT CTA ATG GAA GAT TAT GTG GGC AAC CCG GTG GTA ACC AAT AGA ACA  
 ATA GAT TAC CTT CTA ATA CAC CCG TTG GGC CAC CAT TGG TTA TCT TGT  
 R  
 Tyr Leu Met Glu Asp Tyr Val Gly Asn Pro Val Val Thr Asn Arg Thr>  
 470 \* 480 \* 490 \* 500 \* 510 \*  
 TAT CTC ATG GAG GAT TAC GTG GGC AGC CCC GTG GTG GCG AAC AGA ACA  
 ATA GAG TAC CTC CTA ATG CAC CCG TCG GGC CAC CAC CGC TTG TCT TGT  
 H  
 Tyr Leu Met Glu Asp Tyr Val Gly Ser Pro Val Val Ala Asn Arg Thr>

FIG. 5D

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490 \* 500 \* 510 \* 520 \* 530 \* R  
 TCA CCA CGG AGG AAA CGC TAT GCA GAG CAT AAG AGT CAC CGA GGA GAG  
 AGT GGT GCC TCC TTT GCG ATA CGT CTC GTA TTC TCA GTG GCT CCT CTC

Ser Pro Arg Arg Lys Arg

Tyr Ala Glu His Lys Ser His Arg Gly Glu>  
 MATURE\_\_>>

520 \* 530 \* 540 \* 550 \* H  
 TCA --- CGG CGG AAA CGG TAC GCG GAG CAT AAG AGT CAC CGA GGG GAG  
 AGT --- GCC GCC TTT GCC ATG CGC CTC GTA TTC TCA GTG GCT CCC CTC

Ser \_\_\_ Arg Arg Lys Arg

Tyr Ala Glu His Lys Ser His Arg Gly Glu>  
 MATURE\_\_>>

540 \* 550 \* 560 \* 570 \* 580 \* R  
 TAC TCA GTG TGT GAC AGT GAG AGC CTG TGG GTG ACC GAC AAG TCC TCA  
 ATG AGT CAC ACA CTG TCA CTC TCG GAC ACC CAC TGG CTG TTC AGG AGT

Tyr Ser Val Cys Asp Ser Glu Ser Leu Trp Val Thr Asp Lys Ser Ser>

560 \* 570 \* 580 \* 590 \* 600 \* H  
 TAC TCG GTA TGT GAC AGT GAG AGT CTG TGG GTG ACC GAC AAG TCA TCG  
 ATG AGC CAT ACA CTG TCA CTC TCA GAC ACC CAC TGG CTG TTC AGT AGC

Tyr Ser Val Cys Asp Ser Glu Ser Leu Trp Val Thr Asp Lys Ser Ser>

FIG. 5E

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590	*	600	*	610	*	620	*	630	*	R					
GCC	ATT	GAC	ATT	CGG	GGA	CAC	CAG	GTT	ACA	GTG	TTG	GGA	GAG	ATC	AAA
CGG	TAA	CTG	TAA	GCC	CCT	GIG	GTC	CAA	TGT	CAC	AAC	CCT	CTC	TAG	TTT
Ala Ile Asp Ile Arg Gly His Gln Val Thr Val Leu Gly Glu Ile Lys>															
610	*	620	*	630	*	640	*	650	*	H					
GCC	ATC	GAC	ATT	CGG	GGA	CAC	CAG	GTC	ACG	GTG	CTG	GGG	GAG	ATC	AAA
CGG	TAG	CTG	TAA	GCC	CCT	GTC	GTC	CAG	TGC	CAC	GAC	CCC	CTC	TAG	TTT
Ala Ile Asp Ile Arg Gly His Gln Val Thr Val Leu Gly Glu Ile Lys>															
640	*	650	*	660	*	670	*	680	*	R					
ACC	GGC	AAC	TCT	CCT	GTG	AAA	CAA	TAT	TTT	TAT	GAA	ACG	AGG	TGT	AAA
TGG	CCG	TTG	AGA	GGA	CAC	TTT	GTT	ATA	AAA	ATA	CTT	TGC	TCC	ACA	TTT
Thr Gly Asn Ser Pro Val Lys Gln Tyr Phe Tyr Glu Thr Arg Cys Lys>															
660	*	670	*	680	*	690	*	700	*	H					
ACG	GGC	AAC	TCT	CCC	GTC	AAA	CAA	TAT	TTT	TAT	GAA	ACG	CGA	TGT	AAG
TGC	CCG	TTG	AGA	GGA	CAG	TTT	GTT	ATA	AAA	ATA	CTT	TGC	GCT	ACA	TTC
Thr Gly Asn Ser Pro Val Lys Gln Tyr Phe Tyr Glu Thr Arg Cys Lys>															

FIG. 5F



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780 *      790 *      800 *      810 *      820 *
TCA GAA AAC AAC AAA CTC GTA GGC TGG CGC TGG ATA CGA ATA GAC ACT
AGT CTT TTG TTG TTT GAG CAT CCG ACC GCG ACC TAT GCT TAT CTG TGA      R

Ser Glu Asn Asn Lys Leu Val Gly Trp Arg Trp Ile Arg Ile Asp Thr>

800 *      810 *      820 *      830 *      840 *
TCA GAG AAC AAT AAA CTC GTG GGC TGG CGG TGG ATA CGG ATA GAC ACG
AGT CTC TTG TTA TTT GAG CAC CCG ACC GCG ACC TAT GCC TAT CTG TGC      H

Ser Glu Asn Asn Lys Leu Val Gly Trp Arg Trp Ile Arg Ile Asp Thr>
100

830 *      840 *      850 *      860 *
TCC TGT GTG TGT GCC TTG TCA AGA AAA ATC GGA AGA ACA TGA      R
AGG ACA CAC ACA CGG AAC AGT TCT TTT TAG CCT TCT TGT ACT

Ser Cys Val Cys Ala Leu Ser Arg Lys Ile Gly Arg Thr End>

850 *      860 *      870 *      880 *
TCC TGT GTG TCT GCC TTG TCG AGA AAA ATC GGA AGA ACA TGA      H
AGG ACA CAC ACA CGG AAC AGC TCT TTT TAG CCT TCT TGT ACT

Ser Cys Val Cys Ala Leu Ser Arg Lys Ile Gly Arg Thr End>
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FIG. 5H

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870	*	880	*	890	*	900	*	910	*	920	*	R
ATTGGCATCT	GTCCCCACAT	ATAAATTATT	ACTTTAAATT	ATATGATATG	CAT							
TAACCGTAGA	CAGGGGIGTA	TATTTAATAA	TGAAATTTAA	TATACTATAC	GTA							
890	*	900	*	910	*	920	*	930	*	940	*	H
ATTGGCATCT	CTCCCCATAT	ATAAATTATT	ACTTTAAATT	ATATGATATG	CAT							
TAACCGTAGA	GAGGGGTATA	TATTTAATAA	TGAAATTTAA	TATACTATAC	GTA							
930	*	940	*	950	*	960	*	970	*	980	*	R
GTAGCATATA	AATGTTTATA	TTGTTTTTAT	ATATTATAAG	TTGACCCTTTA	TTTATTAAAC							
CATCGTATAT	TTACAAATAT	AACAAAATA	TATAATATTC	AACTGGAAAT	AAATAATTTG							
950	*	960	*	970	*	980	*	990	*	1000	*	H
GTAGCATATA	AATGTTTATA	TTGTTTTTAT	ATAT-ATAAG	TTGACCCTTTA	TTTATTAAAC							
CATCGTATAT	TTACAAATAT	AACAAAATA	TATA-TATTC	AACTGGAAAT	AAATAATTTG							

FIG. 51

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990	*	1000	1010	1020	1030	1040	1040	*	R
TTCAGCAACC	CTTACAGTAT	ATAAGCTTTT	TTTTCTCAAT	AAAATTGCGT	TGCTTGCCCTT				
AAGTCGTTGG	GAATGTCATA	TATTCGAAAA	AAAAGAGTTA	TTTTAAGCAC	ACGAACGGAA				
1010	*	1020	1030	1040	1050				
TTCAGCAACC	CT-ACAGTAT	ATAAGCTTAT	CGATACCGTC	GACCTCGAGG	GGGGGCC/				H
AAGTCGTTGG	GA-TGTCATA	TATTCGAATA	GCTATGGCAG	CTGGAGCTCC	CCCCCGG/				
1050	*	1060	1070	1080	1090	1100			
CGCTCAGGCC	TCTCCCATCT	GTAAACCTTG	TTTTGTGATT	GGGCTCTCGG	GAACCTTCTG				R
GCGAGTCCGG	AGAGGGTAGA	CAATTGGAAC	AAACACTAA	CCCGAGAGCC	CTTGGAAGAC				
1110	*	1120	1130	1140					
TAAACCTGT	GTACACCAGT	ATTGGCATT	CAGTATTGTC	AA/					R
ATTTGGACA	CATGTGGTCA	TAAACCGTAA	GTCATAACAG	TT/					

FIG. 5J

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5'GG GTC CTG AGC GAC ACG ACC CCC TTG GAG CCC CCG  
3' CG CC CAG GAC GAC TCG CTG TGC TGG GGG AAC CTC GGG GGC

CCC TTG TAT CTC ATG GAG CAT TAC GTG GGC AGC CCC  
GGG AAC ATA GAG TAC CTC CTA ATG CAC CCG TCG GGG

GTG GTG GCG AAC AGA ACA TCA CGG CGG AAA 3'  
CAC CAC CGC TTG TCT TGT AGT GCC GCC TTT GC5'

FIG. 6

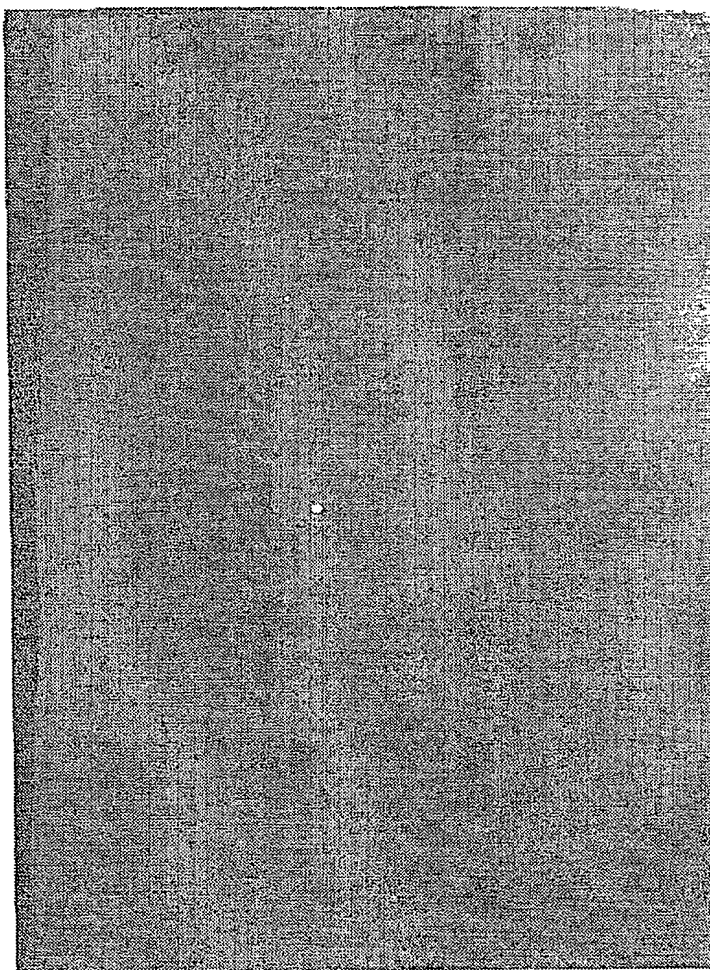
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FIG. 7

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20



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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US92/09792

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(S) : C07K 13/00; C12N 15/18, 1/21, 15/67  
US CL : 530/399; 536/23.5; 435/69.1, 252.3, 320.1

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/399; 536/23.5; 435/69.1, 252.3, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Dialog (Medline, Patents)

search terms: chimera, prepro, neurotroph

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. OF CELL BIOLOGY, Volume 108, No. 5, issued May 1989, T.J. Stoller et al., "The Propeptide of Preprosomatostatin Mediates Intracellular Transport and Secretion of alpha-Globin from Mammalian Cells", pages 1647-1656, see abstract.	1-50
Y	J. OF FERM AND BIOENG, Volume 68, No. 4, issued 1989, H. Oyama, et al., "Secretion of <i>Escherichia coli</i> Aminopeptidase P in <i>Bacillus subtilis</i> Using the Prepro-Structure Coding Region of Subtilisin Amylosacchariticus", pages 289-292, see abstract.	1-50
Y	PROC NATL ACAD SCI, USA, Volume 80, No 23, issued December 1983, S.D. Emr, "An MFalpha1-SUC2 (alpha-factor-invertase) gene fusion for study of protein localization and gene expression in yeast", pages 7080-7084, see abstract.	1-50
Y	DNA, Volume 7, No. 9, issued November 1988, B.R. Cullen, "Expression of a Cloned Human Interleukin-2 cDNA Is Enhanced by the Substitution of a Heterologous mRNA Leader Region", pages 645-650, see abstract.	1-50

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be part of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

11 February 1993

Date of mailing of the international search report

26 FEB 1993

Name and mailing address of the ISA/  
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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US92/09792

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,923,808 (Matteucci) 08 May 1990, col. 1, line 55 - col. 2, line 14.	1-50

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US92/09792

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

- I. Claims 1-15, drawn to a chimeric protein, classified in Class 530/399.
- II. Claims 16-50, drawn to a DNA molecule, a vector, a host cell, and recombinant methods of making the protein, classified in Class 536/23.5 and Class 435/320.1, 69.1, 252.3.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims. (Telephone Practice)
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.